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Clarification of the Systematics of the Haploporoidea (Trematoda) with Descriptions of New Genera and Species

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CLARIFICATION OF THE SYSTEMATICS OF THE HAPLOPOROIDEA (TREMATODA) WITH DESCRIPTIONS OF NEW GENERA AND SPECIES

by

Michael Jay Andres

Abstract of a Dissertation
Submitted to the Graduate School
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ABSTRACT

CLARIFICATION OF THE SYSTEMATICS OF THE

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The superfamily Haploporoidea Nicoll, 1914 comprises two families, the Atractotrematidae Yamaguti, 1939 and the Haploporidae Nicoll, 1914, and the subfamily Cadenatellinae Gibson et Bray, 1982. All members are parasites of the alimentary tract or gall bladder of marine, estuarine, and freshwater herbivorous fishes. Overstreet and Curran (2005a) provided a review of the Haploporidae and recognized four subfamilies, the Chalcinotrematinae Overstreet et Curran, 2005, Haploporinae Nicoll, 1914, Megasoleninae Manter, 1935, and Waretrematinae Srivastava, 1937. In a series of five publications, Blasco-Costa et al. (2009a-e) revised the Haploporinae and erected a fifth subfamily, the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, et Olson, 2009. The present work tests the monophyly of the Haploporinae and Megasoleninae; clarifies the systematics of the Haploporinae, Forticulcitinae, and the Haploporoidea; describes ten new species; erects three new genera and a new family; and provides the first mitochondrial hypotheses for members of the superfamily. A new genus is erected for *Lecithobotrys brisbanensis* (Martin, 1974) that was previously considered a *species inquirenda* and represents the first haploporine coupled with molecular sequence data from outside of the Mediterranean Sea. Two new

species of *Forticulcita* Overstreet, 1982 are described from the New World and a new genus is erected for the two New World species of *Dicrogaster* Looss, 1902. Bayesian inference (BI) analysis suggests that the Forticulcitinae has a New World origin. Three additional species of *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009 are described from Australia; BI analysis resolved the genus as the sister group to the Mediterranean haploporines. A new Indo-Pacific genus is erected for four new species from off Australia, three species originally described in *Haploporus* Looss, 1902, and one species originally described in *Saccocoelium* Looss, 1902. A new species of *Megasolena* Linton, 1910 is described. A BI hypothesis including data for four megasolenines resolved the family Haploporidae and the subfamily Megasoleninae as paraphyletic. Therefore, the Megasoleninae is elevated to the Megasolenidae Manter, 1935 for members with two testes; Cadenatellinae is elevated to the Cadenatellidae Gibson et Bray, 1982; and a new family is erected for genera with a single testis that Overstreet and Curran (2005a) previously considered to belong in the Megasoleninae. Novel mitochondrial (mt) DNA sequence data are provided for members of the Haploporoidea. Concatenated phylogenetic analysis of one mt and two nuclear gene regions support Manter's (1957) hypothesis suggesting that species of *Mugil* Linnaeus have acted as 'ecological bridges' in the radiation of the haploporids.

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A Dissertation
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December 2014

DEDICATION

As I have gone through the process of earning this doctoral degree, my wife, family, and friends have provided support, patience, and understanding. I am forever indebted to them.

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This dissertation is not intended as a scientific record. (see article 8.2, ICZN, International Code of Zoological Nomenclature) for the taxonomic names and nomenclatural acts contained within the dissertation under article 8.3 of the ICZN. This dissertation is not a contribution to the primary scientific literature nor should it be cited as such.

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CHAPTER I

INTRODUCTION

The digenean superfamily Haploporoidea Nicoll, 1914 comprises two families, the Atractotrematidae Yamaguti, 1939 and the Haploporidae Nicoll, 1914 (Jones 2005). Members of the superfamily utilize the alimentary tract or gall bladder of marine (Atractotrematidae and Haploporidae), estuarine (Atractotrematidae and Haploporidae), and freshwater (Haploporidae) herbivorous and omnivorous fishes (Overstreet and Curran 2005a, b). Members of the trematode superfamily are morphologically united by the presence of a hermaphroditic sac enclosing the terminal portion of the male and female reproductive structures but are differentiated by all the atractotrematids possessing two symmetrical or slightly oblique testes. Olson et al. (2003) generated a molecular hypothesis for the Trematoda utilizing complete 18S and partial 28S rDNA sequences of X trematode species, including two species of atractotrematids and a single haploporid, *Hapladena nasonis* Yamaguti, 1970. They recovered the three haploporoid species as a monophyletic clade and transferred both families into the superfamily Gorgoderoidea Looss, 1901 (Xiphidiata Olson, Cribb, Tkach, Bray et Littlewood, 2003), but remarked that the two families were among the most labile. Curran et al. (2006) utilized the 28S rDNA for *Saccocoelioides* sp. for a phylogeny that included 19 other ‘xiphidiatan’ taxa to conclude that the atractotrematids and haploporids are best accommodated by the Haploporoidea.

Members of the Atractotrematidae are united in possessing two symmetrical or slightly oblique testes; interconnected, elongate, lobed vitelline follicles; and a Y-shaped excretory vesicle (Overstreet and Curran 2005b). Because members of the family possess a hermaphroditic sac, the Atractotrematidae has been considered a junior synonym of the Haploporidae (Durio and Manter 1969, Ahmad 1985). However, that view has not prevailed as Yamaguti (1971) and Overstreet and Curran (2005a, b) considered them separate. However, the latter authors considered the status of the Atractotrematidae as tentative, as the family is depauperate and has yet to have a lifecycle published. They considered the family to include four genera, *Atractotrema* Goto et Ozaki, 1929, *Isorchis* Durio et Manter, 1969, *Pseudisorchis* Ahmad, 1985, and *Pseudomegasolena* Machida et Kamiya, 1976, with the latter two not being considered in the family prior to their revision. Olson et al. (2003) demonstrated that *Atractotrema sigani* Durio et Manter, 1969 and *Pseudomegasolena ishigakiense* Machida et Kamiya, 1976 were each other's closest relative and formed a monophyletic clade with *H. nasonis*. Blasco-Costa et al. (2009a), Pulis and Overstreet (2013), Bray et al. (2014), Besprozvannykh et al. (2014), and Andres et al. (2014a) all recovered the two atractotrematids as the sister to the haploporids using 28S rDNA sequence data. While the type species of the Atractotrematidae, *A. sigani*, has a representative sequence, members of the other two genera lack published molecular data.

Overstreet and Curran's (2005a) review of the Haploporidae, with a key to the subfamilies and genera, constitutes the most thorough treatment of the

family. They considered the Megasolenidae Manter, 1935, Waretrematidae Srivastava, 1937, and Hyporhamphitrematidae Machida et Kuramochi, 2000 as junior synonyms of the Haploporidae, and recognized members of the Megasolenidae and Waretrematidae at the subfamily level. They recognized four subfamilies based on morphological characters. They proposed Chalcinotrematinae Overstreet et Curran, 2005, Haploporinae Nicoll, 1914 (syns. Dicrogasterinae Yamaguti, 1958 and Unisaccinae Martin, 1973), Megasoleninae Manter, 1935 (syn. Scorpidicolinae Yamaguti, 1971), and Waretrematinae Srivastava, 1937 (syns. Carassotrematinae Skrjabin, 1942, Spiritestinae Yamaguti, 1958, and Phanurinae Liu et Yang, 2002). Overstreet and Curran (2005a) considered members of the Chalcinotrematinae to possess an extensive uterus (occupying much of the hindbody and often extending into the forebody); either irregularly elongate vitelline follicles that surround the testis or follicles that are irregularly dispersed in hindbody; and hosts primarily estuarine and freshwater fishes in the New World and Africa. The haploporines are united by having a reduced vitellarium; a uterus extending into the hindbody; and members that primarily infect mugilids worldwide (Overstreet and Curran 2005a, Blasco-Costa et al. 2009d). Overstreet and Curran (2005a) regarded the megasolenines to be united by having a confined uterus (extending from ovary to hermaphroditic sac); extensively distributed vitelline follicles; a robust tegument; one or two testis; a cylindrical external seminal vesicle; and a host which is primarily a marine, reef associated perciforme. They considered members of the Waretrematinae to be united by having extensive vitelline follicles; a confined

uterus; a delicate tegument; and a host fish from marine, estuarine, and freshwater habitats worldwide but primarily in the Indo-Pacific Region.

Blasco-Costa et al. (2009a) provided the first molecular framework for the haploporids utilizing 28S rDNA sequences from haploporids isolated from Mediterranean Sea mullets. They erected a fifth subfamily, the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, et Olson, 2009 based on the non-monophyly of the Haploporinae when *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 was included. Their analysis was the first treatment of the family which coupled morphology with molecular data, and casted doubt on the morphological framework of the family by Overstreet and Curran (2005). They considered the subfamily to be united by an eversible ejaculatory duct; a single, coalesced vitellarium; and a mugilid host in the Red and Mediterranean Sea. However, Blasco-Costa et al. (2009c) maintained *Dicrogaster fastigata* Thatcher et Sparks, 1958 as a haploporine, despite that species also possessing a single, coalesced vitellarium.

Pulis and Overstreet (2013) used 28S rDNA sequences to demonstrate that waretrematines with an ornamented oral sucker (e.g., *Waretrema* [sensu Overstreet and Curran 2005a]) was not monophyletic, suggesting that the morphological characters used to establish some genera may be more plastic than previously thought. Pulis et al. (2013) provided sequences of the internal transcribed spacer region (ITS1-5.8S-ITS2) and partial 28S rDNA for two species of the waretrematine *Intromugil* Overstreet et Curran, 2005. Although they did not provide a phylogenetic hypothesis, they stated that *Intromugil* would likely be

closer to members of the Chalcinotrematinae than to waretrematine species. This hypothesis was supported in three recent phylogenetic analyses that included haploporids (Bray et al. 2014, Besprozvannykh et al. 2014, Andres et al. 2014a). Bray et al. (2014) used 28S rDNA sequence data to demonstrate that the enenterid *Cadenatella* Dollfus, 1946, placed in the subfamily Cadenatellinae Gibson et Bray, 1982, belonged within the superfamily Haploporoidea Nicoll, 1914, despite the absence of a hermaphroditic sac in its members. They suggested that the terminal genitalia of *Cadenatella* was derived from the loss of the hermaphroditic sac wall. Nahhas and Cable (1964) were the first to suggest a close association of *Cadenatella* with the haploporids, and that suggestion was based on a lobed oral sucker, an uroproct, single testis, and a kyphosid host.

The four molecular studies discussed above (Blasco-Costa et al. 2009a, Pulis and Overstreet 2013, Pulis et al. 2013, Bray et al. 2014) demonstrate the convergence of some of the morphological characters that had been used for delineating haploporid taxa. Those studies show that the haploporids still require additional examination.

The goals of this dissertation involve the testing of the monophyly of the Haploporinae and clarifying the systematics among members of both the Haploporidae and Haploporoidea. To achieve the first goal, I use a combination of morphological and molecular data to determine the phylogenetic affinity of one species considered by Blasco-Costa et al. (2009b) to be a *species inquirenda* (Chapter II), a species that possess morphological characters inconsistent with its generic diagnosis (Chapter III), a haploporine genus that lacks molecular data

(Chapter IV), and a species transferred by Blasco-Costa et al. (2009e) into a genus formerly allocated to another superfamily (Chapter V). To clarify the systematics among members of the Haploporidae, molecular data are provided for Haploporinae (Chapters II, IV, V); for two new species and a new genus of Forticulcitinae (Chapter III); and for a new species plus three additional members of Megasoleninae, two species of Cadenatellinae, and one species of Atractotrematidae (Chapter VI). Finally, mitochondrial DNA data are used in conjunction with rDNA sequences to clarify the systematics of the Haploporoidea (Chapter VII).

CHAPTER II

ERECTION OF THE TREMATODE *LITOSACCUS* GEN. N. AND ITS PHYLOGENETIC RELATIONSHIP WITHIN THE HAPLOPORIDAE NICOLL, 1914 (TREMATODA)

Abstract

Litosaccus gen. n. is erected for *Paralecithobotrys brisbanensis* Martin, 1974 n. comb. for which an amended description is given. The new genus is morphologically similar to the haploporine *Lecithobotrys* Looss, 1902 but with a more elongate and cylindrical body; an infundibuliform oral sucker; a thin-walled hermaphroditic sac; a shallow genital atrium; and unequal, cylindrical, and elongated caeca. It also resembles *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga, et Kostadinova, 2009, but the only member of that genus has a hermaphroditic sac that is twice the length of the ventral sucker, a hermaphroditic duct with intensely-staining cuboidal cells, an elongate testis, and single caecum or paired caeca. A Bayesian inference analysis of partial 28S rDNA sequences of *L. brisbanensis* and 24 other haploporoids revealed that *L. brisbanensis* grouped with other haploporines and placed *Intromugil* Overstreet et Curran, 2005 in a clade with the chalcinotrematine *Saccocoelioides* Szidat, 1954 rather than with the other seven tested waretrematine species. This analysis represents the first phylogenetic study of the Haploporidae Nicoll, 1914 that incorporates a haploporine from outside of the Mediterranean Sea.

Introduction

Martin (1974) described the haploporid *Paralecithobotrys brisbanensis* Martin, 1974 from the Brisbane River, Queensland (QLD), Australia, in *Mugil cephalus* Linnaeus. In a review of the Haploporidae Nicoll, 1914, Overstreet and Curran (2005a) reported that the holotype of *P. brisbanensis* had been temporarily lost, but they examined specimens of *P. brisbanensis* collected by RMO from the type-host, near the type-locality. They transferred *P. brisbanensis* to *Lecithobotrys* Looss, 1902 as *Lecithobotrys brisbanensis* (Martin, 1974) because members of *Paralecithobotrys* Teixeira de Freitas, 1947 have vitelline follicles distributed in a patchy manner rather than in two distinct, grape-like clusters (as in *Lecithobotrys*) and are found in non-mugilid, freshwater fishes in South America and Africa. Additionally, they considered *Paralecithobotrys* to belong in the subfamily Chalcinotrematinae Overstreet et Curran, 2005. Blasco-Costa et al. (2009b) revised *Haploporus* Looss, 1902 and *Lecithobotrys* and considered *L. brisbanensis* to be a *species inquirenda*. They considered it to possess morphological features inconsistent with *Lecithobotrys*, namely an elongate cylindrical body, a weakly-muscularised genital atrium, a poorly-developed hermaphroditic sac, and an armed hermaphroditic duct. Citing the loss of the type material and morphological differences between *Lecithobotrys* and *L. brisbanensis* sp. inq., Blasco-Costa et al. (2009b) suggested that description of new material from the type-host and type-locality was needed to assess the generic affiliation of *L. brisbanensis*.

Blasco-Costa et al. (2009a) provided the first molecular phylogenetic hypothesis for the Haploporidae based on sequences of partial 28S ribosomal DNA (rDNA), and it included the type-species of *Lecithobotrys*, *Lecithobotrys putrescens* Looss, 1902, and eight other haploporine genera. Since then, four additional works on haploporids have incorporated molecular data. Pulis and Overstreet (2013) generated the second molecular hypothesis for the family and included four waretrematines. Pulis et al. (2013) described *Intromugil alachuaensis* Pulis, Fayton, Curran, et Overstreet, 2013 and provided sequences of the internal transcribed spacer region (ITS1-5.8S-ITS2) and partial 28S rDNA for two species of *Intromugil* Overstreet et Curran, 2005. Besprozvannykh et al. (2014) restored *Parasaccocoelium* Zhukov, 1971 and resolved three species of that genus close to the waretrematine genus *Capitimitta* Pulis et Overstreet, 2013 based on analysis of partial 28S rDNA sequence data. Bray et al. (2014) used the same gene region to demonstrate that *Cadenatella* Dollfus, 1946 belongs within the superfamily Haploporoidea Nicoll, 1914, despite the absence of a hermaphroditic sac in its members, for which they used subfamily name Cadenatellinae Gibson et Bray, 1982. Here I report on freshly collected specimens of *L. brisbanensis* from the type-host near the type-locality, provide supplemental material, and present a Bayesian inference (BI) analysis of partial 28S rDNA sequences to test its phylogenetic placement within the Haploporidae.

Materials and Methods

During March, 2010, three moribund specimens resembling *L. brisbanensis* sp. inq. were collected from *M. cephalus* cast-netted off Shorncliffe,

Queensland (QLD), Australia, following the method of Cribb and Bray (2010) for gastrointestinal species, but skipping the initial examination under a dissecting microscope because of the large volume of intestinal contents. The worms were rinsed and cleaned in a container with saline and examined briefly; then, most of the saline was decanted, the worms were killed by pouring hot (not boiling) water over them, and they were fixed in 70% ethanol. Additional specimens of *L. brisbanensis* sp. inq. were collected from *M. cephalus* during: April, 1984, off Redland Bay, QLD, January, 1995, from the Brisbane River, Toowong, QLD, and November, 1997, from off Shorncliffe, and Wynnum Creek, QLD. Worms were stained in Mayer's haematoxylin or Van Cleave's haematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Canada balsam (Van Cleave's) or Dammar gum (Mayer's). Measurements were made using a compound microscope equipped with a differential interference contrast, a Canon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres and data for the illustrated specimen are followed by the range of data for the other specimens in parentheses. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis and Overstreet (2013).

Genomic DNA was isolated from two entire specimens using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,550 base pairs (bp) long, comprising the 3' end of the 18S nuclear rRNA gene, internal transcribed spacer region

(including ITS1 + 5.8S + ITS2), and the 5' end of the 28S rRNA gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction (PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAA GTT G-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3'), and the internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer's instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences from the species were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Sequences obtained from GenBank are as follows: *Atractotrema sigani* Durio et Manter,1969 (AY222267) (Olson et al. 2003), *Cadenatella isuzumi* Machida, 1993 (FJ788497) (Bray et al. 2009), *Cadenatella pacifica* (Yamaguti, 1970) (FJ788498) (Bray et al. 2009), *Capitimitta costata* Pulis et Overstreet, 2013 (KC206497) (Pulis and Overstreet 2013), *Capitimitta darwinensis* Pulis et Overstreet, 2013 (KC206498) (Pulis and Overstreet 2013), *Capitimitta* sp.

(KC206499) of Pulis and Overstreet (2013), *Dicrogaster contracta* Looss, 1902 (FJ211262) (Blasco-Costa et al. 2009a), *Dicrogaster perpusilla* Looss, 1902 (FJ211238) (Blasco-Costa et al. 2009a), *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 (FJ211239) (Blasco-Costa et al. 2009a), *Hapladena nasonis* Yamaguti, 1970 (AY222265) (Olson et al. 2003), *Haploporus benedeni* Looss, 1902 (FJ211237) (Blasco-Costa et al. 2009a), *Intromugil alachuaensis* (KC430095) (Pulis et al. 2013), *Intromugil mugilicolus* (Shireman, 1964) (KC430096) (Pulis et al. 2013), *L. putrescens* (FJ211236) (Blasco-Costa et al. 2009a), *Litosaccus brisbanensis* (Martin, 1974) (KM253765) (Andres et al. 2014a), *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko, et Nikitenko, 2014 (HF548461) (Besprozvannykh et al. 2014), *Parasaccocoelium mugili* Zhukov, 1971 (HF548468) (Besprozvannykh et al. 2014), *Parasaccocoelium polyovum* (HF548474) (Besprozvannykh et al. 2014), *Pseudomegasolena ishigakiense* Machida et Kamiya, 1976 (AY222266) (Olson et al. 2003), *Saccocoelioides* sp. of Curran et al. (2006) (EF032696) (Curran et al. 2006), *Saccocoelium brayi* Blasco-Costa, Montero, Balbuena, Raga, Kostadinova, et Olson, 2009 (FJ211234) (Blasco-Costa et al. 2009a), *Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009 (FJ211233) (Blasco-Costa et al. 2009a), *Saccocoelium obesum* Looss, 1902 (FJ211260) (Blasco-Costa et al. 2009a), *Saccocoelium tensum* Looss, 1902 (FJ211258) (Blasco-Costa et al. 2009a), and *Spiritestis herveyensis* Pulis et Overstreet, 2013 (KC206500) (Pulis and Overstreet 2013). The sequences were aligned using MAFFT version 6.611b (Katoh et al. 2005)

with 1,000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). The resulting alignment utilised two atractotrematids, two species of *Cadenatella*, and 22 haploporids with the paragonimid *Paragonimus westermani* (Kerbert, 1878) as the outgroup based on its phylogenetic position relative to the Haploporoidea (Olson et al. 2003). Phylogenetic analysis of the data was performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba et al. 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The following model parameters were used in MrBayes: nst = 6, rates = invgamma, ngen = 1,000,000 and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al. 2001) with all other settings left as default.

Results

Litosaccus gen. n.

Diagnosis. Body of adult elongate, cylindrical, slightly more than 6× longer than wide. Tegument sparsely spinous. Eye-spot pigment diffuse in forebody. Oral sucker terminal, infundibuliform, with small papillae surrounding periphery.

Ventral sucker slightly elevated, transversely oval, shorter than oral sucker. Prepharynx distinct. Pharynx subglobular to globular, smaller than oral sucker. Oesophagus present. Intestinal bifurcation approximately at second fifth of body length. Caeca two, cylindrical, uneven to subequal, end blindly at approximately last quarter of body. Testis single, subspherical, median, located approximately at level of midbody. External seminal vesicle claviform to sac-like. Hermaphroditic sac not well-developed, in first quarter of body length, arcuate, elongate-oval, slightly longer than to 1.5× length of pharynx; sac containing internal seminal vesicle, small prostatic bulb, thin walled male duct, female duct, and hermaphroditic duct. Genital atrium shallow. Ovary subglobular to globular, medial, pretesticular. Uterus occupies most of hindbody. Vitellarium in two clusters of subglobular to globular follicles, posterolateral to ovary. Eggs numerous, containing developed miracidia with two fused eye-spots. Excretory vesicle I-shaped, bulbous anteriorly, terminating in hindbody. In Mugilidae; in Southwest Pacific Region.

Type and only species: *Paralecithobotrys brisbanensis* Martin, 1974.

Etymology: The Greek *litos* for 'simple' and the masculine Greek *saccus* for 'sac' refer to the small, relatively simple hermaphroditic sac.

Remarks. The new genus presently accommodates only *Litosaccus brisbanensis* (Martin, 1974) n. comb. that is morphologically most similar to the haploporine genera *Lecithobotrys* and *Pseudolecithobotrys* Blasco-Costa, Gibson, Balbuena, Raga, et Kostadinova, 2009 in possessing a vitellarium comprising two grape-like clusters of follicles lateral to the ovary. The new genus

can be separated from the two by possessing two uneven caeca, an infundibuliform oral sucker, a small, thin-walled hermaphroditic sac (hermaphroditic sac length/ ventral sucker length 57–104% as opposed to over 110%), and shallow genital atrium. Additionally, it can be further differentiated from *Lecithobotrys* in having an elongate, cylindrical body rather than a fusiform to pyriform body and can be further differentiated from *Pseudolecithobotrys* in possessing a subspherical testis rather than an elongate, subcylindrical testis. Martin (1974) originally described *P. brisbanensis* as having a hermaphroditic duct "lined with tiny spines or tubercles" (18), a feature I cannot confirm. The specimens I examined do not appear to have any spines or tubercles lining the hermaphroditic duct, although he stated that it is best seen in specimens with an everted duct, not present in the specimens examined.

Litosaccus brisbanensis (Martin, 1964) n. comb. Figure. 2.1.

syns. *Paralecithobotrys brisbanensis* Martin, 1964; *Lecithobotrys brisbanensis* (Martin, 1964) Overstreet et Curran, 2005

Description (measurements based on 11 gravid wholemounts): Body elongate, cylindrical, 2,048 (1,416–2,256) long, 302 (227–285) wide at second fifth of body length (BL), with width representing 15 (12–19)% of BL. Tegumental spines exceptionally thin, 5–10 (6–13) long. Forebody 563 (339–581) long, representing 27 (23-30)% of BL. Hindbody 1,312 (923–1,575) long, representing 64 (60–70)% of BL. Oral sucker infundibuliform, terminal, 259 (192–267) long, 245 (201–234) wide, with anterior periphery surrounded by ring of approximately 12 small papillae. Ventral sucker 173 (154–192) long, 204 (137–190) wide. Ratio

of oral sucker to ventral sucker width 1:0.83 (1:0.67–0.88). Prepharynx 64 (41–88) long. Pharynx subglobular, approximately twice length of prepharynx, 118 (89–128) long, 126 (99–121) wide. Ratio of oral sucker width to pharynx width 1:0.51 (1:0.48-0.60). Oesophagus 96 (117–317) long, extending to second fifth of BL, swollen posteriorly. Intestinal bifurcation at or posterior to level of ventral sucker. Caeca long, relatively narrow, uneven to subequal (sinistral caecum longer in all but 1 specimen), more bulbous posteriorly in most specimens, terminating blindly, with posterior-most caecum terminating 481 (293–577) from posterior end, with postcaecal space representing 24 (15-34)% of BL.

Testis single, 151 (113–211) long, 129 (113–163) wide, 270 (210–346) from posterior margin of ventral sucker. Post-testicular space 893 (443–1,074) long, representing 44 (28–48)% of BL. External seminal vesicle claviform to sac-like, 163 (72–158) long, 68 (29–75) wide, dorsal to ventral sucker. Hermaphroditic sac thin-walled, anterodorsal to dorsal of ventral sucker, 112 (109–190) long, 67 (55–89) wide, representing 65 (57–104)% of ventral sucker length and 5 (6–10)% of BL, containing internal seminal vesicle 78 (61–102) long by 38 (24–40) wide, prostatic bulb, female duct, and hermaphroditic duct; male and female ducts unite at anterior third of hermaphroditic sac; hermaphroditic duct muscularised, approximately 1/3 length of hermaphroditic sac. Genital pore medial, 55 (10–56) anterior to anterior margin of ventral sucker.

Ovary globular to subglobular, medial, 91 (67–145) long, 94 (65–109) wide, 101 (17–130) from posterior margin of ventral sucker, 76 (9–227) from anterior margin of testis, posteroventral to ventral to intestinal bifurcation. Uterus

emerging from dextral side of ovary, winding anteriorly to or slightly beyond posterior margin of ventral sucker and then winding posteriorly, occupying most of hindbody, with proximal portion filled with sperm. Laurer's canal not observed. Vitellarium in 2 lateral clusters of 7–10 subglobular to spherical follicles 26–30 (24–46) long by 26–29 (23–39) wide, with sinistral cluster 125 (96–162) long, dextral cluster 103 (79–129) long, contiguous or nearly so with posterior margin of ovary, with anterior-most follicle 157 (106–218) from posterior margin of ventral sucker, ventral to caeca. Eggs thin-shelled, numerous, with those in distal portion of uterus mostly with developed miracidia having eyespots fused, 40–45 (40–46) long, 24–26 (22–26) wide.

Excretory vesicle I-shaped, bulbous anteriorly, terminating immediately posterior to ovary, with 1 specimen having well-defined crura extending anteriorly from level of vitelline clusters; pore terminal.

Type and only known host: *Mugil cephalus* Linnaeus, flathead grey mullet (Mugilidae).

Type-locality: Brisbane River, Queensland, Australia.

Other localities: Shorncliffe Beach, Bramble Bay, QLD, 27°19'26"S, 153°5'10"E (Figure 2.1A); Shorncliffe Boat Ramp, Cabbage Tree Creek, QLD, 27°19'47"S, 153°5'11"E (DNA); Brisbane River, Toowong, QLD (27°29' 29"S, 152°59'34"E); Wynnum Creek, QLD (27°26'9"S, 153°10'28"E); Redland Bay, QLD.

Site: Intestine.

Type-material: Hancock Parasitology Collection, University of Southern California, No. 7112 (presently unable to locate).

Voucher material: Queensland Museum, Brisbane, Australia 234515-22; Harold W. Manter Laboratory Collection, Lincoln, Nebraska, USA P-2014-021.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. KM253765, from 2 identical sequences (2 adult specimens from Cabbage Tree Creek, QLD).

Figure 2.1. *Litosaccus brisbanensis* n. comb. from *Mugil cephalus.* A. Ventral view. B. Ventral view of tegumental spines in sinistral margin of forebody. C. Lateral view of hermaphroditic sac. D. Ventral view of other specimens showing caecal variation. Scale-bars: A., D. 500 µm; B.,C. 50 µm.

Remarks. Martin's (1974) type material (originally deposited in the no longer cohesive Hancock Parasitology Collection, University of Southern California) is still missing; I have been unsuccessful in my attempt to find the holotype at the Santa Barbara Museum of Natural History (Pers. comm. Daniel Geiger and Patricia Sadeghian), the Los Angeles County Museum of Natural History (Pers. comm. Joel Martin), and the U.S. National Helminthological Collection (Pers. comm. Patricia Pilitt). For consistency I chose to illustrate and measure the same specimen illustrated by Overstreet and Curran (2005a) in their chapter in the *Keys to the Trematoda* Vol. 2 (Figure 12.9). The excretory vesicle was described by Martin (1974) as being Y-shaped, but it is I-shaped in all specimens I examined. However, in one of the specimens, the one illustrated (Figure 2.1A), there are well-defined crura extending from level of the vitelline clusters. These crura are likely collecting branches because each is differentiated from the vesicle by a sphincter. Martin (1974) did not indicate the presence of small papillae surrounding the oral sucker that usually are apparent on many well-fixed trematodes, but the shape of the oral sucker in his illustration and his measurements are consistent with the specimens treated herein. Martin (1974) reported the tegument as mostly smooth but with a few spines dorso-anteriorly and immediately posterior to the ventral sucker. Tegumental spines were observed by in only four of the specimens; two had thin spines sparsely covering the entire tegument and two had only a few spines posterior to the ventral sucker. Presumably, the spines of *L. brisbanensis* are fragile, shallowly embedded, or easily lost and were therefore not observed on most of the

specimens because of loss due to fixation, preservation, or handling techniques. Despite these potential differences and based on the size and shape of the body, suckers, reproductive organs, and hermaphroditic sac, I have no doubt that the specimens collected are conspecific with those of Martin (1974).

Molecular analysis

The DNA sequence fragment amplified encompasses the 3' end of the 18S gene, the ITS region (ITS1-5.8S-ITS2) and 1,415 bp of the 5' end of the 28S gene. No intraspecific variation occurred between the two sequenced specimens of *L. brisbanensis*. The alignment of partial 28S rDNA sequences of *L. brisbanensis* and related species from GenBank was 1,128 characters long with 655 conserved sites, 473 variable sites, and 337 informative sites. The BI analysis of those sequences incorporated the paragonimid *P. westermani* as an outgroup and an ingroup of two species each of atractotrematids and *Cadenatella*, *L. brisbanensis*, and 21 other species of Haploporidae (Figure 2.2). The ingroup of the Haploporidae was revealed as a paraphyletic clade. The megasolenine *Hapladena nasonis* Yamaguti, 1970 was well supported as basal to *Cadenatella* spp. and the other haploporids. The position of *Cadenatella* as sister to the non-*Hapladena* haploporids was poorly supported. The 20 other non-*Hapladena* haploporids formed a polytomy consisting of *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009, *Spiritestis herveyensis* Pulis et Overstreet, 2013, *Capitimitta* spp. + *Parasaccocoelium* spp., and a clade that included two subclades: one comprised of *Intromugil* spp. +

Saccocoelioides sp. and the other of *Litosaccus brisbanensis* + the Mediterranean haploporines.

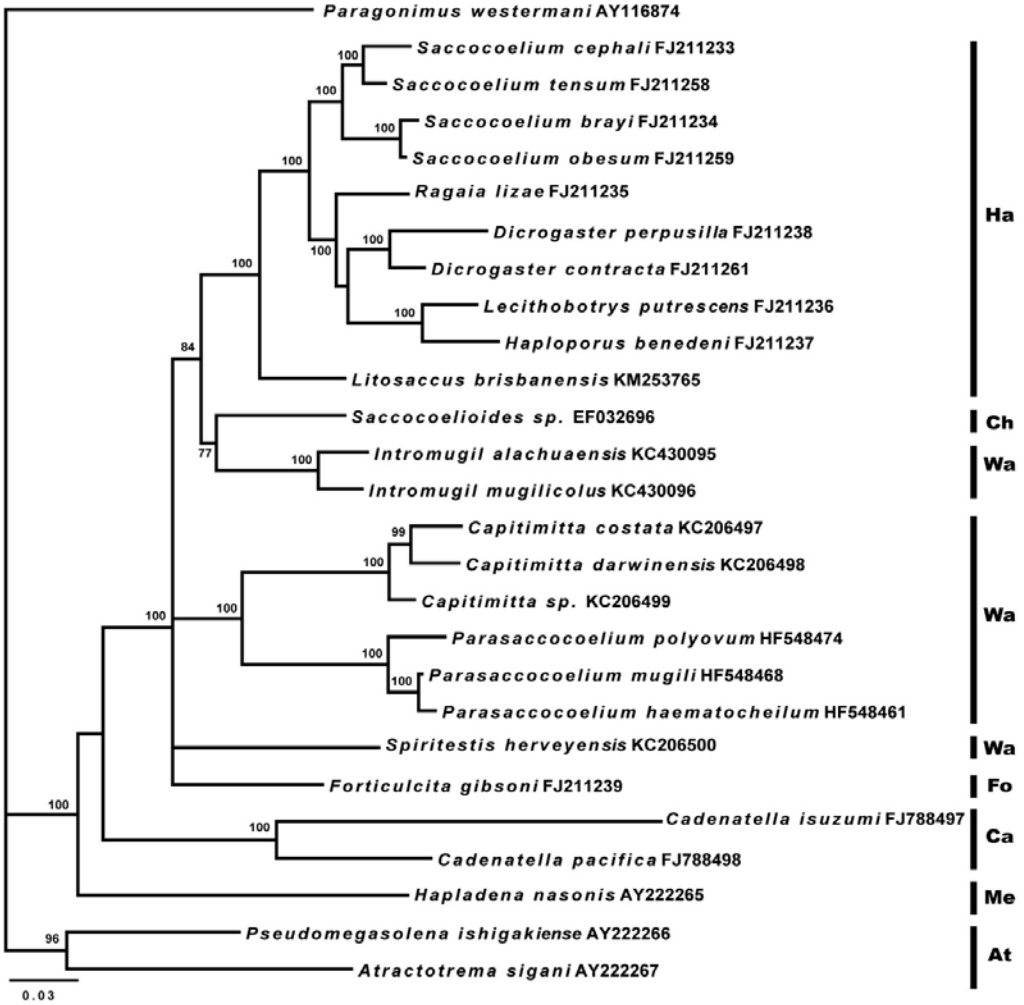


Figure 2.2. Phylogenetic relationships among members of the Haploporidae resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100) revealing *Litosaccus brisbanensis* n. comb as a haploporine. Support values of <75% not shown. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellinae; Ch = Chalcinotrematinae; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasoleninae; Wa = Waretrematinae.

Discussion

Blasco-Costa et al. (2009b) considered *Lecithobotrys brisbanensis* as a *species inquirenda* and stated that it likely did not belong in *Lecithobotrys*; my BI analysis confirms that it does not. *Litosaccus* was erected for *L. brisbanensis*,

which has morphological characters in common with the Haploporinae (i.e., vitellarium that is reduced, a uterus that occupies much of the hindbody but does not extend into the forebody, and developed eggs containing miracidia with eye-spots) and is similar to *Lecithobotrys* and *Pseudolecithobotrys*.

In view of the only slight morphological discrepancies between Martin's (1974) specimens and those treated herein, I have little doubt that the specimens are conspecific with those originally described. In the redescription of *I. mugilicolus* by Pulis et al. (2013), they noted that the hermaphroditic duct had a "series of sacs containing a glandular substance" (502) that was observable in living specimens and specimens stored in ethanol, but they were no longer easily discernible after processing for mounting. Similarly, the "tiny spines or tubercles" (18) described by Martin (1974) as lining the hermaphroditic duct of *L. brisbanensis* may not be apparent in the fixed specimens I examined. Thus, additional specimens need to be examined live to confirm the presence or absence of an armed hermaphroditic duct. *Litosaccus* is not an appropriate repository for either of the other two species of *Lecithobotrys* considered *species inquirenda* by Blasco-Costa et al. (2009b), and I agree that both require further data to clarify their generic affinity.

To the best of my knowledge, *L. brisbanensis* may be considered rare or its host has not been collected when the infection is at its peak intensity. A total of 46 specimens of *M. cephalus* from the QLD coast (12 in 1984, 18 in 1997, and 16 in 2010) have been examined and only a total of 16 specimens were recovered, all from the Brisbane/ Moreton Bay area. Lester et al. (2009) found

that approximately 50% of the individuals of *M. cephalus* they examined had evidence of infection by the blood fluke *Plethorchis acanthus* Martin, 1975 in the Moreton Bay area, while *M. cephalus* from along the New South Wales coast showed no such infection, suggesting the parasite was acquired in Moreton Bay, perhaps in the upper estuary. A similar pattern may occur for infection with *L. brisbanensis*, because the parasite was only recovered from Moreton Bay drainages. Additionally, in 2010, 65 individuals of the greenback mullet, *Chelon subviridis* (Valenciennes), flat-tail mullet, *Liza argentea* (Quoy et Gaimard), and silver mullet, *Paramugil georgii* (Ogilby), were examined from Cabbage Tree Creek and the Pine River, which, along with the Brisbane River, empty into Moreton Bay, and no specimen of *L. brisbanensis* was recovered.

In a review of the Haploporidae, Overstreet and Curran (2005a) recognized four subfamilies based on morphology: the Chalcinotrematinae (infecting estuarine and freshwater fishes in the New World and Africa), the Haploporinae (members with primarily in mugilids worldwide), the Megasoleninae Manter, 1935 (primarily in marine, reef associated perciformes) and the Waretrematinae Srivastava, 1937 (in marine, estuarine, and freshwater fishes worldwide, but primarily in the Indo-pacific). Blasco-Costa et al. (2009a) established the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, et Olson, 2009 (with members in mugilids in the Mediterranean Sea and Red Sea) based on a single, compact vitellarium and their BI analysis of partial 28S rDNA sequence data. This is the first phylogenetic hypothesis of Haploporidae to include a haploporine collected outside of the Mediterranean Sea. *Litosaccus*

was resolved as distinct from *Lecithobotrys* but well supported as sister to the Mediterranean haploporines (Figure 2.2), confirming that members of the Haploporinae are not restricted to the Mediterranean Sea.

I agree with Pulis and Overstreet's (2013) skepticism of the morphologically defined haploporid subfamilies due to the paucity of molecular data for most genera. My BI analysis revealed the Waretrematinae to be paraphyletic with *Intromugil* being closer to *Saccocoelioides* Szidat, 1954 and *Spiritestis* Nagaty, 1948 being recovered in the polytomy leading to the other major haploporid clades, but, at this time, I refrain from making any nomenclatural changes. Besprozvannykh et al. (2014) resurrected *Parasaccocoelium* and demonstrated that the three species they treated formed a well-supported clade with *Capitimitta*, which I recovered as well. However, I am skeptical of their consideration of *Pseudohapladena lizae* Liu et Yang, 2002 as a junior synonym of *Parasaccocoelium mugili* Zhukov, 1971. Liu and Yang (2002) described *Ps. lizae* as having a longer oesophagus, smaller eggs, a well-separated ovary and testis, and a more tubular vitellarium.

Bray et al. (2014) used BI analysis of 28S rDNA sequences to demonstrate that *Cadenatella* had previously been misplaced in the Enenteridae Yamaguti, 1958 (Lepocreadioidea Odhner, 1905) and belongs in the Haploporoidea. They noted that with the inclusion of *Cadenatella* in the Haploporoidea, the Haploporidae was not well resolved because *Hapladena* Linton, 1910 did not cluster with the other members of the family. I also resolved *Hapladena* (the sole representative of the Megasoleninae included in both

analyses) outside of the clade containing *Cadenatella* spp. and the rest of the haploporids. The position of *Cadenatella* as the sister group to the rest of the haploporids was not well supported; thus, an important component of future considerations will be whether these taxa belong in the Haploporidae or whether there is a case for recognition of further family level taxa within the Haploporoidea.

The systematics of haploporids still requires considerable resolution. Erecting *Litosaccus* brings the total number of haploporine genera to ten. Four of those genera, *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009, *Pseudolecithobotrys*, *Rondotrema* Thatcher, 1999, and *Unisaccus* Martin, 1973, lack a representative DNA sequence. Since all four of those genera also lack a Mediterranean representative, their inclusion in a molecular framework will help clarify the subfamilial relationships within the Haploporidae and help detect the pattern of diversification within the Haploporinae.

CHAPTER III

AN ADDITIONAL GENUS AND TWO ADDITIONAL SPECIES OF FORTICULCITINAE (TREMATODA: HAPLOPORIDAE NICOLL, 1914)

Abstract

Forticulcita sp. n. 1 and *Forticulcita* sp. n. 2 are described from *Mugil liza* Valenciennes in Argentina, and from *Mugil cephalus* Linnaeus in Salt Springs, Florida, USA, respectively. Supplemental material relating to the hermaphroditic sac of *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 is provided from a specimen isolated from *M. cephalus* off Crete, Greece. *Forticulcita* sp. n. 1 can be distinguished from all species of *Forticulcita* Overstreet, 1982, except *F. gibsoni*, based on possessing small pads or gland cells along the hermaphroditic duct. It can be differentiated from that species in possessing a hermaphroditic sac that is one and a half to two times longer than wide rather than one that is approximately three times longer than wide and in possessing larger eggs (44-52 µm long by 20-26 µm wide rather than 34-44 µm long by 18-24 µm wide). *Forticulcita* sp. n. 2 can be differentiated from the other species of *Forticulcita* in possessing a testis that is shorter than or equal to the pharynx rather than one that is longer than the pharynx. *G*en. n. 1 is erected for *Dicrogaster fastigata* Thatcher et Sparks, 1958, as Gen. n. 1 *fastigta* (Thatcher et Sparks, 1958) comb. n. The new genus fits within the concept of Forticulcitinae Blasco-Costa, Balbuena, Kostadinova et Olson, 2009 in having a vitellarium comprised of a single elongate to subspherical mass. Gen. n. 1 can be differentiated from *Forticulcita* in having spines lining the hermaphroditic duct. A

Bayesian inference analysis of the 28S rDNA of the two New World species of *Forticulcita*, Gen. n. 1 *fastigata*, and previously published haploporids places Gen. n. 1 *fastigata* within the Forticulcitinae and sister to *Forticulcita*. Amended diagnoses for the subfamily and for *Dicrogaster* Looss, 1902 are provided.

Introduction

Overstreet (1982) erected *Forticulcita* Overstreet, 1982 for *Forticulcita glabra* Overstreet, 1982 from the bluespot mullet, *Moolgarda seheli* (Forsskål) (as *Valamugil seheli* [Forsskål]), off Eilat, Israel, in the Red Sea. He considered *F. glabra* to be closest to members of *Haploporus* Looss, 1902 rather than to *Dicrogaster* Looss, 1902 because members of *Dicrogaster* have an armed hermaphroditic duct and 'characteristically stubby' caeca relative to those of *F. glabra*. Hassanine (2007) described a second species, *Forticulcita mugilis* Hassanine, 2007 from *Crenimugil crenilabis* (Forsskål) also in the Red Sea off Sharm El-Sheikh, South Sinai, Egypt. Blasco-Costa et al. (2009b) described *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 from *Mugil cephalus* Linnaeus off Santa Pola, Spain. Blasco-Costa et al. (2009a) established the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, et Olson, 2009 based on the presence of a 'well-delimited eversible intromittent copulatory organ' or 'ejaculatory organ', vitellarium a single spherical to subtriangular compact mass, and a Bayesian inference (BI) hypothesis of the partial 28S rDNA that resolved *F. gibsoni* outside of the Haploporinae Nicoll, 1914.

Dicrogaster fastigata Thatcher et Sparks, 1958 has been the only accepted non-forticulcitine haploporid with a vitellarium comprised of a single elongate to subspherical mass of follicles. Yamaguti (1958) erected the Dicrogasterinae Yamaguti, 1958 for the members of *Dicrogaster*. He considered the key character of the subfamily to be the single median vitellarium. Overstreet (1982) did not accept the Dicrogasterinae (that action was corroborated in the molecular hypothesis by Blasco-Costa et al. [2009a]) because the type-species, *Dicrogaster perpusilla* Looss, 1902, and *Dicrogaster contracta* Looss, 1902 were both described as possessing a vitellarium comprised of two close subspherical masses. Fernández Bargiela (1987) described another species with a single vitellarium, *Dicrogaster fragilis* Fernández Bargiela, 1987 from *M. cephalus* off Chile. Blasco-Costa et al. (2009b) revised *Dicrogaster*, considered *D. fragilis* to be a junior synonym of *D. fastigata*, and accepted only *D. perpusilla*, *D. contracta*, and *D. fastigata*. In this study, I provide the description of two New World species of *Forticulcita*, provide supplemental material relating to the hermaphroditic duct of *F. gibsoni*, erect a new genus to accept *D. fastigata*, and provide a BI analysis based on partial 28S rDNA that estimates the affinity of those species with 26 previously published haploporoids.

Materials and Methods

Specimens of *M. cephalus* were collected from Grand Isle, Louisiana, USA, in June, 2013, and Davis Bayou, Ocean Springs, Mississippi, USA, in March 2010 using a cast-net. Thomas Fayton collected specimens of *M. cephalus* from Salt Spring, Florida, USA, in March, 2013, by Hawaiian sling.

Specimens of *Mugil liza* Valenciennes were collected from Rio de la Plata, Punta Lara, and Rio Salado, Cerro de la Gloria, both in Provincia de Bueno Aires, Argentina, in March, 2008, using a cast-net. Ronnie Palmer collected a single *M. cephalus* from Chania, Crete, Greece, in September, 2005, by baited dip-net. Trematodes were collected following the procedure outlined by Cribb and Bray (2010) for gastrointestinal species, skipping the initial examination under a dissecting scope because of the high volume of the intestinal contents. Worms were rinsed and cleaned in a container with saline and examined briefly. Subsequently, most of the saline was removed from the container, and the worms were killed by pouring near-boiling water over them (with the exception of a single worm collected from Chania that was fixed under coverslip pressure with a lighter) and then preserved in 70% ethanol or 10% buffered formalin. Worms were stained in Van Cleave's haematoxylin or Mayer's haematoxylin, dehydrated in a graded ethanol series, cleared in clove oil (Van Cleave's) or methyl salicylate (Mayer's), and mounted permanently in Canada balsam (Van Cleave's) or Dammar gum (Mayer's). Measurements were made using a compound microscope equipped with a differential interference contrast, a Canon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres; data for the holotype are followed by the range of data for the other specimens in parentheses. Terminology pertaining to the hermaphroditic sac and its structures follows the terminology by Pulis and Overstreet (2013). The museum collection abbreviations are used as follows: BMNH, British Museum of Natural History, London, England; USNM,

Smithsonian National Museum of Natural History, Washington, DC, USA; and USNPC, United States National Parasite Collection (previously in Beltsville, Maryland, USA.). Representative specimens will be submitted to museums before the chapter is submitted for publication, thus collection numbers for new material are listed as to be determined (TBD).

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,550 base pairs (bp) long, comprising the 3' end of the 18S nuclear rDNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction (PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3'), and the internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2(5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI

BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 5.0) and representative sequences submitted to GenBank. Sequences obtained from GenBank are as follows: *Atractotrema sigani* Durio et Manter,1969 (AY222267) (Olson et al. 2003), *Cadenatella isuzumi* Machida, 1993 (FJ788497) (Bray et al. 2009), *Cadentella pacifica* (Yamaguti, 1970) (FJ788498) (Bray et al. 2009), *Capitimitta costata* Pulis et Overstreet, 2013 (KC206497) (Pulis and Overstreet 2013), *Capitimitta darwinensis* Pulis et Overstreet, 2013 (KC206498) (Pulis and Overstreet 2013), *Capitimitta* sp. (KC206499) of Pulis and Overstreet (2013), *D. contracta* (FJ211262) (Blasco-Costa et al. 2009a), *D. perpusilla* (FJ211238) (Blasco-Costa et al. 2009a), *F. gibsoni* (FJ211239) (Blasco-Costa et al. 2009a), *Hapladena nasonis* Yamaguti, 1970 (AY222265) (Olson et al. 2003), *Haploporus benedeni* Looss, 1902 (FJ211237) (Blasco-Costa et al. 2009a), *Intromugil alachuaensis* Pulis, Fayton, Curran, et Overstreet, 2013 (KC430095) (Pulis et al. 2013), *Intromugil mugilicolus* (Shireman, 1964) (KC430096) (Pulis et al. 2013), *Lecithobotrys putrescens* Looss, 1902 (FJ211236) (Blasco-Costa et al. 2009a), *Litosaccus brisbanensis* (Martin, 1974) (KM253765) (Andres et al. 2014a), *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko, et Nikitenko, 2014 (HF548461) (Besprozvannykh et al. 2014), *Parasaccocoelium mugili* Zhukov, 1971 (HF548468) (Besprozvannykh et al. 2014), *Parasaccocoelium polyovum* (HF548474) (Besprozvannykh et al. 2014),

Pseudomegasolena ishigakiense Machida et Kamiya, 1976 (AY222266) (Olson et al. 2003), *Saccocoelioides* sp. of Curran et al. (2006) (EF032696) (Curran et al. 2006), *Saccocoelium brayi* Blasco-Costa, Montero, Balbuena, Raga, Kostadinova, et Olson, 2009 (FJ211234) (Blasco-Costa et al. 2009a), *Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009 (FJ211233) (Blasco-Costa et al. 2009a), *Saccocoelium obesum* Looss, 1902 (FJ211260) (Blasco-Costa et al. 2009a), *Saccocoelium tensum* Looss, 1902 (FJ211258) (Blasco-Costa et al. 2009a), and *Spiritestis herveyensis* Pulis et Overstreet, 2013 (KC206500) (Pulis and Overstreet 2013). The sequences were aligned using MAFFT version 6.611b (Katoh et al. 2005) with 1000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). Phylogenetic analysis of the data was performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba et al. 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The following model parameters were used in MrBayes: nst = 6, rates = invgamma, ngen = 1,000,000 and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500),

and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al. 2001) with all other settings left as default.

Results

Forticulcita gibsoni Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009

Type and only known host: *Mugil cephalus* Linnaeus, flathead grey mullet, Mugilidae.

Type-locality: Off Santa Pola, Spain ($38^{o}00'$-$38^{o}20'$N, $0^{o}10'$-$0^{o}40'$E).

Other localities: Ebro Delta, Spain ($40^{o}30'$-$40^{o}50'$N, $0^{o}30'$-$1^{o}10'$E); Phalasarna, Crete, Greece ($35^{o}30'07''$N, $23^{o}34'37''$E).

Site: Intestine.

Holotype: BMNH 2008.10.7.61.

Material examined: Paratypes (12 specimens) BMNH 2008.10.7.62-76; 1 flattened specimen BMNH TBD.

Supplemental material: Hermaphroditic duct lined with pads or gland cells (Figure 3.1).

Remarks. The pads or gland cells lining the hermaphroditic duct of *F. gibsoni* were not apparent in the paratypes, but based on morphological features and geographic locations I have no reason not to consider the specimen collected by Ronnie Palmer conspecific with *F. gibsoni*. Additional measurements are not presented as the specimen was fixed under coverslip pressure.

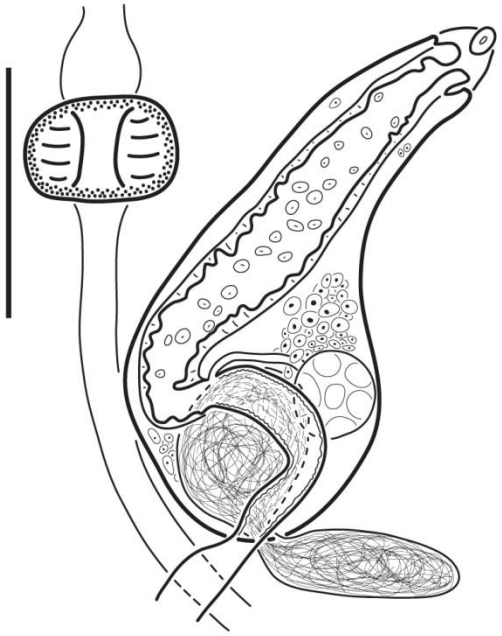


Figure 3.1. Hermaphroditic sac of flattened specimen of *Forticulcita gibsoni* displaying pads or gland cells lining hermaphroditic duct. Scale bar: 100 µm.

Forticulcita sp. n. 1 Figure 3.2A-B.

Description (measurements based on 17 mature wholemounts): Body fusiform, 790 (501-745) long, widest at midbody,188 (131-214) wide representing 24% (24-29%) of body length (BL). Forebody 197 (154-198) long representing 25% (23-31%) BL. Hindbody 496 (279-471) long representing 63% (56-64%) of BL. Eyespot pigment dispersed in anterior 2/3 of forebody. Tegumental spines stubby, 1-2 long, occurring over entire surface, becoming sparse in posterior 1/4 to 1/3 of body. Oral sucker transversely subspherical, subterminal, 79 (71-81) long, 95 (73-100) wide. Ventral sucker subspherical 94 (68-97) long, 97 (64-92) wide. Ratio of oral sucker to ventral sucker widths 1: 1.02 (0.74-0.95). Prepharynx 28 (41-68) long. Pharynx transversely subglobular, 47 (89-120) long, 56 (99-121) wide. Ratio of oral sucker width to pharyngeal width 1: 0.59 (1: 0.53-0.68). Oesophagus 263 (176-222) long, extending to approximately midbody.

Intestinal bifucation 99 (26-127) posterior to level of ventral sucker. Caeca sac-like, approximately twice as long as wide, vacuolar, terminating blindly 278 (164-369) from posterior end, postcaecal space representing 35% (33-50%) of BL.

Testis single, elongate to subspherical, 101 (59-101) long, 52 (42-65) wide, 189 (92-226) from posterior margin of ventral sucker. Posttesticular space 20% (19-36%) of BL. External seminal vesicle claviform, 60 (34-63) long, 27 (17-23) wide, dorsal to ventral sucker. Hermaphroditic sac kidney bean-shaped, 154 (120-171) long, 92 (55-89) wide representing 19% (20-24%) of BL, containing oval to spherical internal seminal vesicle measuring 65 (32-63) long by 64 (26-56) wide in posterior portion with swollen prostatic bulb 51 (38-51) long by 47 (36-59) wide and with short male duct uniting with female duct at approximately midlevel of sac; hermaphroditic duct heavily muscular, approximately 1/2 length of hermaphroditic sac, lined with ovoid pads or cells of an uncertain function. Genital atrium shallow; genital pore medial, 27 (13-26) anterior to anterior margin of ventral sucker.

Ovary spherical to elongate, 68 (64-86) long, 60 (33-66) wide, 109 (27-203) from posterior margin of ventral sucker, contiguous with testis to 80 (3-38) from anterior margin of testis, anteroventral or ventral to intestinal bifurcation. Laurer's canal opening dorsally at level of intestinal bifurcation to level of anterior margin of ventral sucker. True seminal receptacle lacking. Vitellarium a single spherical to elongate mass, 61 (40-65) long, 60 (36-63) wide, 173 (87-153) from posterior margin of ventral sucker, dorsal to and contiguous with testis, mostly intercaecal. Uterus occupying most of hindbody, with proximal portion filled with

sperm. Eggs in distal portion of uterus 51-52 (44-52) long, 24-26 (20-26) wide; eggs of most specimens in distal portion of uterus containing developed miracidia having 2 separate to fused eyespots; 1 specimen with hatched miracidia in hermaphroditic duct.

Excretory vesicle weakly Y-shaped (femur-shaped), terminating at level immediately posterior to or at posterior margin of testis, representing approximately 25% (22-33%) of BL; pore terminal.

Type and only known host: *Mugil liza* Valenciennes, Lebranche mullet, Mugilidae.

Site: Intestine.

Type-locality: Rio de la Plata, Punta Lara, Provincia de Buenos Aires, Argentina (34^{o}49'04"S, 57^{o}58'03"W).

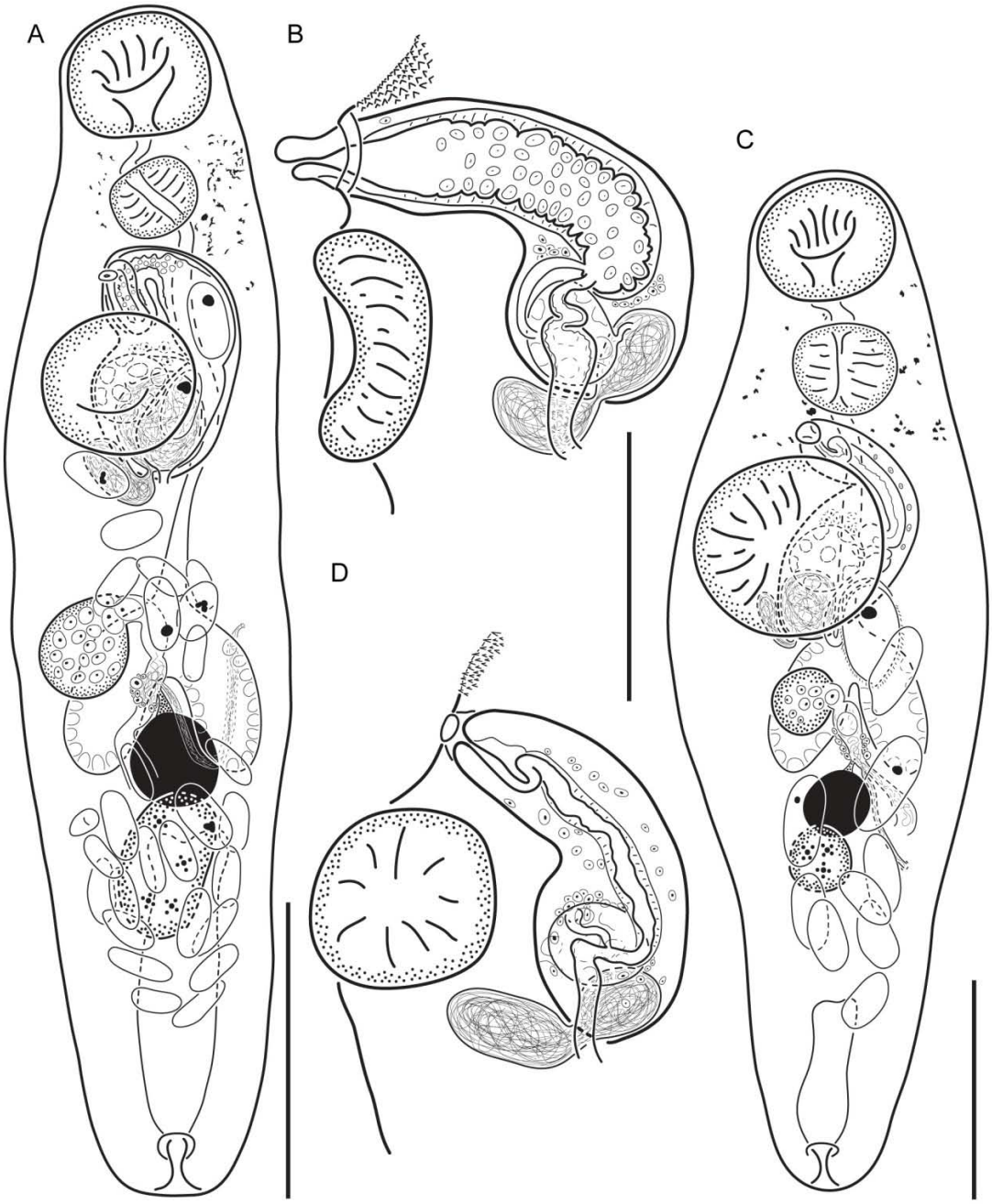
Other locality: Rio Salado, Cerro de la Gloria, Provincia de Buenos Aires, Argentina (35^{o}58'23"S, 57^{o}26'57"W).

Specimens deposited: Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina, holotype (TBD); paratypes TBD, USNM TBD, and BMNH TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (SSC-23) from 2 identical sequences (2 adult specimens; 1 from Rio de la Plata and 1 from Rio Salado).

Remarks. *Forticulcita* sp. n. 1 can be distinguished from all other species of *Forticulcita* except *F. gibsoni* based on a hermaphroditic duct lined with pads or gland cells. The new species is morphologically most similar to *F. gibsoni*, but it can be differentiated from that species in possessing a hermaphroditic sac that

is one and a half to two times longer than wide rather than approximately three times longer than wide, eggs that are slightly larger (44-52 µm long X 20-26 µm wide rather than 34-44 µm long by 18-24 µm wide).



Figures 3.2. A-B. *Forticulcita* sp. n. 1. A. Ventral view of holotype. B. Lateral view of hermaphroditic sac and stubby tegumental spines. C-D. *Forticulcita* sp. n. 2. C. Ventral view of holotype. D. Lateral view of hermaphroditic sac and thin tegumental spines. Scale bars: A. 200 µm; C. 100 µm; B,D. 100 µm.

Forticulcita sp. n. 2 Figures 3.2D-C.

Description (*measurement based on 9 mature wholemounts*): Body fusiform, 469 (354-524) long, widest at midbody,148 (124-153) wide representing 32% (28-35%) BL. Forebody 132 (124-158) long representing 28% (28-35%) BL. Hindbody 258 (157-285) long representing 55% (44-55%) of BL. Eyespot pigment dispersed in forebody to level of approximately 1/3 of BL Tegumental spines thin, 2-4 long, located over entire surface, becoming sparse in posterior 1/4 to posterior 1/3 of body. Oral sucker transversely subspherical, subterminal, 53 (45-67) long, 63 (54-72) wide. Ventral sucker spherical, 79 (73-84) long, 86 (81-87) wide. Ratio of oral sucker to ventral sucker widths 1: 0.73 (0.69-0.86). Prepharynx 16 (9-28) long. Pharynx transversely subglobular, 41 (31-46) long, 47 (42-51) wide. Ratio of oral sucker to pharyngeal widths 1: 0.75 (1: 0.63-0.93). Oesophagus 91 (71-121) long, extending to approximately midbody. Intestinal bifurcation at level of approximately middle to immediately posterior to ventral sucker, dorsal to anterodorsal to hermaphroditic sac. Caeca sac-like, approximately twice as long as wide, vacuolar, terminating blindly at 191 (126-212) from posterior end; postcaecal space representing 41% (34-45%) of BL.

Testis single, subspherical to elongate, 35 (31-53) long, 28 (25-36) wide, contiguous with to 82 from posterior margin of ventral sucker. Posttesticular space 133 (96-186) representing 28% (25-36%) of BL. External seminal vesicle claviform, 27 (21-44) long, 14 (12-24) wide, dorsal to ventral sucker, anteriorly directed. Hermaphroditic sac claviform, 99 (84-128) long, 55 (41-55) wide, representing 21% (19-30%) of BL, containing subspherical internal seminal

vesicle measuring 29 (26-36) long by 28 (20-34) wide in posterior portion with swollen prostatic bulb 31 (29-36) long by 33 (24-37) wide and with short male duct uniting with female duct at approximately posterior 1/3 to midlevel of sac; hermaphroditic duct heavily muscular, approximately 1/2 to 2/3 length of hermaphroditic sac. Genital atrium shallow (4-7); genital pore medial, 12 (8-20) anterior to anterior margin of ventral sucker.

Ovary subspherical to elongate, 31 (27-42) long, 28 (22-30) wide, located at level from approximately middle of ventral sucker to 23 (7-67) from its posterior margin, 42 (7-19) from anterior margin of to contiguous with testis, ventral or posteroventral to intestinal bifurcation, ranging from dextral to sinistral. Laurer's canal pore opening dorsally at level of approximately middle of hindbody. True seminal receptacle lacking. Vitellarium a single subspherical to elongated mass, 31 (28-40) long, 30 (24-32) wide, 59 (20-95) from posterior margin of ventral sucker, dorsal to and contiguous with testis, mostly intercaecal. Uterus occupying most of hindbody, with proximal portion filled with sperm. Eggs in distal portion of uterus 48-49 (38-48) long, 21-22 (14-20) wide; developed miracidia having 2 separated or fused eyespots; 1 specimen (holotype) having hatched miracidia in distal portion of uterus.

Excretory vesicle weakly Y-shaped (femur-shaped) to nearly I-shaped when swollen (in 2 specimens), extending to approximately middle level of hindbody, representing approximately 19% (18-29%) of BL; pore terminal.

Type and only known host: *Mugil cephalus* Linnaeus, flathead grey mullet, Mugilidae.

Site: Intestine.

Prevalence of infection: 6 of 8 individuals.

Type-locality: Salt Springs, St. Johns River, Marion County, Florida, USA (29°21'01"N, 81°43'57"W).

Specimens deposited: Holotype, USNM TBD; paratypes USNM TBD, BMNH TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (SJ3-1) from 1 adult specimen.

Remarks. *Forticulcita* sp. n. 2 can be differentiated from the other species of *Forticulcita* based on its overall smaller size and a testis that is shorter than or equal in length to the pharynx. The two new species also differ on a molecular basis.

Gen. n. 1

Diagnosis. Body fusiform. Tegument spinous. Eyespot pigment dispersed, mostly in forebody. Oral sucker subspherical, subterminal. Ventral sucker larger than oral sucker. Forebody approximately as long as body width at level of ventral sucker. Prepharynx present. Pharynx well-developed. Oesophagus approximately 1 to 6 times longer than pharynx. Caeca saccular, terminating blindly at level of approximately middle of hindbody. Testis elongate. External seminal vesicle present. Hermaphroditic sac elongate, approximately twice as long as ventral sucker, approximately twice as long as female duct. Hermaphroditic duct highly muscular, lined with spines. Ovary elongate to subspherical, variably positioned. Vitellarium single mass, elongate to

subspherical. Eggs with developed occulate miracidia. Excretory vesicle Y-shaped; pore terminal.

Type-species: Dicrogaster fastigatus Thatcher et Sparks, 1952

Remarks. Gen. n. 1can be differentiated from all other haploporid genera, with the exception of *Forticulcita*, based on a vitellarium comprised as a single elongate to subspherical mass rather than one that is transversely elongated and dumbbell-shaped as in *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009 or one that consists of two close subpherical masses as in *Dicrogaster*. Gen. n. 1 can be easily differentiated from *Forticulcita* based on possessing a hermaphroditic sac armed with spines

Gen. n. 1 fastigata (Thatcher and Sparks, 1958) comb. n.

syns. Dicrogaster fastigatus Thatcher and Sparks, 1958

Type-host: *Mugil cephalus* Linnaeus, flathead grey mullet, Mugilidae.

Site: Intestine.

Type locality: Grand Isle, Louisiana, USA.

Other localities: Rockefeller Wildlife Refuge, Grand Chenier, Louisiana, USA; waters of and adjacent to Mississippi Sound, Mississippi, USA.

Holotype: USNPC 38389; supplemental material: USNM TBD, BMNH TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-281) from 4 identical sequences (2 adult specimens from Grand Isle, LA, USA [29^{o}14'18"N,

90°0'13"W], and 2 adult specimens from Davis Bayou, Ocean Springs, MS, USA [30°23'31"N, 88°47'55"W]).

Remarks. Fernández Bargiela (1987) described specimens of *D*. *fastigata* from *M. cephalus* off Arica and Concepción, both Chile. She also described *D*. *fragilis* from the same host off Concepción. She considered *D*. *fragilis* to be separate from *D*. *fastigata* based on having a thin, fragile tegument that is mostly devoid of spines; a smaller oral sucker, ventral sucker, and pharynx; and a larger ovary, testis, and hermaphroditic sac. Blasco-Costa et al. (2009 c) considered *D. fragilis* a junior subjective synonym of *D. fastigata* because the morphometrics of *D. fragilis* were within the range of those reported for *D*. *fastigata*. I have not examined specimens of either of the purported species reported by Fernández Bargiela (1987); however, I believe all her specimens of the two taxa to be conspecific. The reported tegumental difference is possibly related to fixation technique or because the specimens of *D*. *fragilis* might have been dead or moribund when removed from the host. However, I am highly skeptical that Gen. n. 1 *fastigata* occurs along the Pacific Coast of the New World. The increased use of molecular techniques has revealed a large number of cryptic helminth species (e.g., Pérez-Ponce de León and Nadler 2010, Blasco-Costa et al. 2010, Poulin 2011), and I think *D. fragilis* is one. I agree with Blasco-Costa et al. (2009c) that all the Chilean specimens are conspecific. However, I consider them all to be Gen. n. 1 *fragilis* until sequence data are collected that would show that no difference existed between Atlantic and Pacific populations.

Molecular analysis

The DNA sequence fragment for *Forticulcita* sp. n. 1 encompasses 120 bp portion in the 3' end of the 18S gene, 599 bp in the ITS1, 157 bp in the 5.8S, 275 bp in the ITS2, and 1,387 bp of the 5' end of the 28S gene. No intraspecific variation was observed in the two specimens sequenced. The DNA sequence fragment for *Forticulcita* sp. n. 2 encompasses 23 bp in the 3' end of the 18S gene, 600 bp in the ITS1, 157 bp in the 5.8S, 271 bp in the ITS2, and 1,387 bp of the 5' end of the 28S gene. The ITS1 sequence of *Forticulcita* sp. n. 1 exhibits 96.2% similarity to (23 bp different from) the ITS1 sequence of *Forticulcita* sp. n. 2. The DNA sequence fragment for Gen. n. 1 *fastigata* encompasses 120 bp in the 3' end of the 18S gene, 904 bp in the ITS1, 157 bp in the 5.8S, 279 bp in the ITS2, and 1,387 bp of the 5' end of the 28S gene. No intraspecific variation was observed in the four specimens sequenced. The ITS1 sequence of Gen. n. 1 *fastigata* is 304 bp longer than that of *Forticulcita* sp. n. 1 and 305 bp longer than that of *Forticulcita* sp. n. 2. Pairwise comparison of the ITS2 and partial 28S of *F. gibsoni* (FJ211262) and the three other new world forticulcitines are found in Table 3.1.

The sequence alignment utilized 2 atractotrematids, 2 species of *Cadenatella* Dollfus, 1946, and 25 haploporids, and it was 1,128 characters long with 663 conserved sites, 465 variable sites, and 335 informative sites. The BI analysis of partial 28S rDNA gene sequences used the atractotrematid *A. sigani* as the outgroup based on its phylogenetic position within the Haploporoidea (Olson et al. 2003, Andres et al. 2014a) and an ingroup containing *Cadenatella*

spp., *Forticulcita* sp. n. 1, *Forticulcita* sp. n. 2, Gen. n. 1 *fastigata*, and 22 other species of Haploporidae (Figure 3.3). The Haploporidae was revealed as

Table 3.1

Pairwise comparisons of percent nucleotide similarity and number of base pair differences (in parentheses) of the ITS-2 (below the diagonal) and 28S (above the diagonal) of the three species of Forticulcita and Gen. n. 1 fastigata.

	F. gibsoni	*F.* sp. n. 1	*F.* sp. n. 2	Gen. n. 1 *fastigata*
F. gibsoni	-	99.6 (5)	98.7 (16)	94.9 (61)
F. sp. n. 1	98.5 (4)	-	98.6 (17)	94.8 (62)
F. sp. n. 2	93.7 (17)	94.5 (15)	-	94.9 (61)
Gen. n. 1 *fastigata*	87.1 (35)	83.5 (45)	85.1 (40)	-

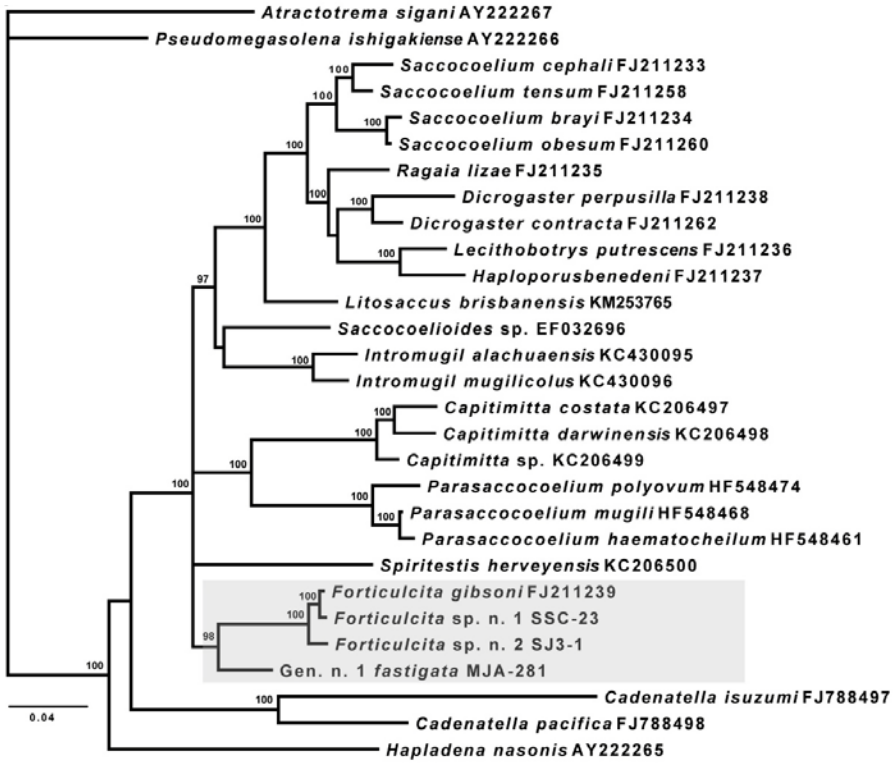


Figure 3.3. Phylogenetic relationships among members of the Haploporidae resulting from Bayesian inference analysis of partial sequences of 28S rDNA gene (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100) revealing a monophyletic Forticulcitinae (shaded rectangle), with Gen. n. 1 *fastigata* n. comb as the sister to *Forticulcita*. Support values of <75 not shown.

paraphyletic as was demonstrated by previous authors (Bray et al. 2014, Andres et al. 2014a), with *H. nasonis* basal to *Cadenatella* and the rest of the Haploporidae. The 22 other non-*Hapladena* haploporids formed a polytomy consisting of the Forticulcitinae, *S. herveyensis*, *Capitimitta* spp. + *Parasaccocoelium* spp., and a clade that included two subclades: one comprised of *Intromugil* spp. and *Saccocoelioides* sp. and the other of the Haploporinae. The Forticulcitinae was well supported with Gen. n. 1 *fastigata* as sister to the three species of *Forticulcita*.

Discussion

Prior to this study, *Forticulcita* contained three species: *F. glabra* (type-species) and *F. mugilis* from the Red Sea (Overstreet 1982, Hassanine 2007) and *F. gibsoni* from the Mediterranean Sea (Blasco-Costa et al. 2009b). The two new species expand the geographic range of the genus to the New World and into freshwater. *Forticulcita* sp. n. 1 can be separated from the other members of *Forticulcita*, with the exception of *F. gibsoni*, in possessing pads or glandular cells along the hermaphroditic duct. *Forticulcita* sp. n. 2 can be separated from the other members of *Forticulcita* based on its smaller size and by having the testicular length shorter than or equal to the pharyngeal length. The three members of *Forticulcita* that have representative DNA sequences are morphologically similar to each other, and they differ from both Red Sea species by having a smaller body length (BL < 1,050), a subspherical rather than triangular to irregular vitellarium, a subspherical rather than an elongate internal seminal vesicle, and a clavate rather than an elongate external seminal vesicle.

Even though I consider the two New World species in *Forticulcita*, I am skeptical that the above characteristics are of specific value and believe that once molecular data become available for *F*. *glabra*, a new genus may be needed to accommodate *F*. *gibsoni*, *Forticulcita* sp. n. 1, and *Forticulcita* sp. n. 2. Additionally, Overstreet's (1982) specimens of *F. glabra* were fixed under slight coverslip pressure, and, after examination of four paratypes and a photograph of the holotype (USNPC 76518), I was unable to determine the shape of the excretory vesicle. Hassanine (2007) reported the excretory vesicle of *F. mugilis* as I-shaped, Blasco-Costa et al. (2009c) reported that the excretory vesicle of *F*. *gibsoni* has a "bifurcation at mid-hindbody" (201), and the subfamily diagnosis by Blasco-Costa et al (2009a) mentions a Y-shaped excretory vesicle. Based on my observation of the excretory vesicle of *Forticulcita* sp. n. 2, I consider the excretory vesicle within *Forticulcita* to be either I- to weakly Y-shaped.

I erected Gen. n. 1 to accommodate Gen. n. 1 *fastigata* based on the possession of a single compact elongate to subspherical vitellarium (a character I consider to be important at the subfamily level), spines lining the hermaphroditic duct, and my BI analysis of the partial 28S rDNA. Based on geographic locality, I tentatively accept Gen. n. 1 *fragilis* as the only other species within Gen. n. 1. Gen. n. 1 *fastigata* was reported by Knoff et al. (1997) in an ecological study of *M. liza* (as *M. platanus* Günther) off Rio de Janeiro, Brazil. They did not deposit any voucher material or offer any descriptive information on the species. Because the report may refer to a new species, I am tentatielvy not considering Gen. n. 1 *fastigata* to extend its range to Rio de Janeiro without confirmation.

Yamaguti (1958) was the first to recognize that a single compact vitellarium could be an important subfamilial character when he erected the Dicrogasterinae. However, because *D*. *perpusilla* has two closely situated masses, Overstreet (1982) did not accept the subfamily and considered *Dicrogaster* a haploporine, which Blasco-Costa et al. (2009a) demonstrated in their molecular analysis. I agree with Blasco-Costa et al.'s (2009c) erection of *Pseudodicrogaster* for *Dicrogaster japonica* Machida, 1996 and agree that it is best placed in the Haploporinae. *Pseudodicrogaster japonica* (Machida, 1996) has a vitellarium that is a compact, transversely elongated, dumb-bell-shaped mass rather than the single subspherical to irregular mass present in members of the Forticulcitinae. I consider the vitellarium being in a single grouping rather than paired (i.e., dumbbell-shaped) to be an important character for the Forticulcitinae. Thus, *Dicrogaster* Looss, 1902 currently contains only *D. perpusilla* and *D. contracta* and the generic diagnosis by Overstreet and Curran (2005a) necessitates the amendment eyespot pigment diffuse but concentrated around the pharynx; oesophagus being equal in size to approximately twice the length of pharynx; testis being located in the median of the hindbody; and the vitelline follicles being coalesced, forming two closely situated masses adjacent to ovary. The addition of these New World species to the Forticulcitinae necessitates an amended subfamily diagnosis to that originally provided by Blasco-Costa et al. (2009a). The diagnosis should now include eye-spot pigment dispersed between oral sucker and mid-hermaphroditic sac; external seminal vesicle clavate to elongate; hermaphroditic sac elongate, kidney bean-shaped to subcylindrical;

hermaphroditic duct eversible, lined with spines or not; ovary pretesticular, contiguous with to separated from testis; excretory vesicle I- to weakly Y-shaped.

The BI analysis presented by Blasco-Costa et al. (2009a) estimated the placement of the Forticulcitinae (based on *F. gibsoni*) as basal to *Saccocoelioides* sp., but my BI analysis could not resolve the placement of the subfamily relative to the other non-megasolenine haploporid subfamilies. The topology of the BI tree is identical to the one presented by Andres et al. (2014a), but with the only meaningful difference being the slightly lower support for the *Saccocoelioides* sp. + *Intromugil* spp. clade, and the slightly higher support for the relationship of that clade with the Haploporinae. Gen. n. 1 *fastigata* was recovered as the sister to *Forticulcita*, with *Forticulcita* sp. n. 2 as the basal member of that genus. My phylogeny suggests that the Forticulcitinae may have a New World origin and that the reduction of the vitellarium has evolved at least twice within the Haploporidae: once within the Haploporinae and once within the Forticulcitinae.

Surprisingly, *Forticulcita* sp. n. 1 is genetically closer to *F. gibsoni* than it is to the other New World species, *Forticulcita* sp. n. 2 (Table 3.1, Figure 3.3). One possible explanation is the isolation of *Forticulcita* sp. n. 2 within a freshwater spring system. Although there is the possibility that *Forticulcita* sp. n. 2 was acquired in estuarine waters and carried with its host to the freshwater spring, I believe that *Forticulcita* sp. n. 2 was acquired in the spring. The hosts from which specimens of *Forticulcita* sp. n. 2 were isolated were collected at Salt Springs, Florida, approximately 120 km from the mouth of the St. John's River.

Additionally, the specific conductance (an indirect measure of salinity) at Salt Springs is higher than that of the rest of the St. John's River, with the exception of coastal and estuarine locations (Scott et al. 2004, St. Johns River Water Management District 2013). The extensive spring systems across the limestone 'dome' of the Florida peninsula provide important habitats for rich biological communities, including obligate spring taxa (Nordlie 1990, Walsh 2001) and marine and estuarine species (Odom 1953, Walsh 2001, Smock et al. 2005). Odum (1953) surveyed the inland distribution of marine organisms on the peninsula of Florida and related their distribution to the relatively high freshwater chlorinity derived from relic salt deposits in the marine limestone of the Floridian aquifer system. In particular, he found that the St. John's River system had the most extensive 'marine invasions' because of the numerous ionic springs that feed into it. The ionic composition of these springs has allowed for the establishment of patches of estuarine species far removed from the mouth of the river (Smock et al. 2005). Finally, during May, 2009, I examined eight specimens of *M. cephalus* from Trout River, Jacksonville, FL (30^{o}24'13"N, 81^{o}39'50"W), near the mouth of the St. John's River, but I found no forticulcitine species. However, additional specimens of *M. cephalus* from other springs and the lower reaches of the St. John's River, along with potential intermediate hosts from Salt Springs, need to be examined to confirm that *Forticulcita* sp. n. 2 is a spring associated species. Pulis et al. (2013) suggested a similar pattern of infection for *Intromugil alachuaensis* collected from the Santa Fe River, Florida.

Although isolation of *Forticulcita* sp. n. 2 in freshwater may explain the slightly larger genetic distance between it and the other two species of *Forticulcita*, the isolation does not help explain the low genetic distance observed between *F. gibsoni* and *Forticulcita* sp. n. 1 (Table 3.1, Figure 3.3). The high genetic similarity between the two species separated by the Atlantic Ocean is surprising, especially considering they are genetically closer to each other than any of the Mediterranean haploporine species are to one another. Of the haploporine species, *Saccocoelium brayi* and *S*. *obesum* are the two most closely related (97.5% similarity in the ITS2 and 99.2% similarity in the 28S), which is slightly less than what I found for *F. gibsoni* and *Forticulcita* sp. n. 1 (Table 3.1). The close genetic similarity between *F*. *gibsoni* and *Forticulcita* sp. n. 1 would seem to suggest that *Forticulcita* was established in the Mediterranean Sea from the New World relatively recently. One such mechanism for the radiation of *Forticulcita* in the Mediterranean Sea could have been accomplished by rafting (e.g., Thiel and Haye 2006). The dispersal of a haploporid by aquatic vegetation rafts could have been achieved by the first intermediate host being carried out on the raft, the final host using the raft for shelter over the open ocean, or as the second intermediate host itself since haploporid cercariae typically encyst on aquatic vegetation. Collection of potential hosts from the west coast of Africa and the Atlantic volcanic islands, such as the Cape Verde Islands, Ascension Island, and St. Helena, may help discern if additional, closely related species of *Forticulcita* occur there and if rafting was a viable explanation. Clearly, molecular data from additional species of *Forticulcita*, in particular the type-

species, *F. glabra*, are needed to help understand the pattern of radiation within the subfamily.

CHAPTER IV

DESCRIPTION OF THREE NEW AUSTRALIAN SPECIES OF *PSEDUODICROGASTER* (TREMATODA: HAPLOPORIDAE NICOLL, 1914) FROM THE SQUARETAIL MULLET, *ELOCHELON VAIGIENSIS* (MUGILIDAE)

Abstract

Three species of *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009 are described from the diamond scale mullet, *Ellochelon vaigiensis* (Quoy et Gaimard, 1825) off Western Australia, Northern Territory, and Queensland, Australia. All three Australian species can be distinguished from *Pseudodicrogaster japonica* (Machida, 1996) in having a hermaphroditic sac comprising approximately 45% of body length rather than approximately 30% of body length and a forebody that is approximately 25% or more of body length rather than less than 20% of body length. *Pseudodicrogaster* sp. n. 1 can be distinguished from the other Australian species by having a prepharynx length less than or equal to the pharynx length, mature eggs longer than 61 µm rather than less than 58 µm, and an internal seminal vesicle that is 1.5 times the length of the external seminal vesicle rather greater than 1.5 times the length of the external seminal vesicle. *Pseudodicrogaster* sp. n. 2 can be distinguished from the other Australian species by possessing a testis length to caeca length ratio less than 1: 1. *Pseudodicrogaster* sp. n. 3 can be distinguished from the other Australian species by possessing caeca that are 5 to 12 times longer than wide and a hermaphroditic duct that is more than three times longer than the external seminal vesicle. My Bayesian inference analysis of partial 28S

rDNA sequences utilized two atractotrematids, two species of *Cadenatella*, three species of *Pseudodicrogaster*, and 35 other haploporids. The three species of *Pseudodicrogaster* were revealed in a monophyletic clade with the other members of the Haploporinae Nicoll, 1914. The three species of *Pseudodicrogaster* formed a monophyletic clade sister to the Mediterranean haploporine species, with *Litosaccus brisbanensis* (Martin, 1974) and as the basal haploporine. An amended diagnosis of *Pseudodicrogaster* is provided and *Rondotrema* Thatcher, 1999 is transferred from the Haploporinae to the Chalcinotrematinae Overstreet et Curran, 2005.

Introduction

Machida (1996) described *Dicrogaster japonica* Machida, 1996 from *Mugil cephalus* Linnaeus off Fukaura, Japan, stating that is was close to *Dicrogaster contracta* Looss, 1902 in having vitellarium composed of two compact masses, but differs from that species in having an internal seminal vesicle that is tubular. Blasco-Costa et al. (2009d) examined Machida's (1996) specimens and considered *D. japonica* to possess characters not in common with Overstreet and Curran's (2005a) diagnosis of *Dicrogaster* Looss, 1902. Namely, a 'dumbbell' shaped vitellarium, pads lining the hermaphroditic duct, tubular internal and external seminal vesicles, and longer caeca (more than twice the length of the ventral sucker). Blasco-Costa et al. (2009d) also discussed the possible close association with *Forticulcita* Overstreet, 1982 based on a compact vitellarium and tubular internal and external seminal vesicle. However, they did not consider *Forticulcita* an appropriate repository based on members of *Forticulcita* having a

fusiform body, an elongate hermaphroditic sac, an internal seminal vesicle much shorter than the external seminal vesicle, and a testis located at the level of the midbody. Thus, they erected *Pseudodicrogaster* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009 for *D. japonica*, as *Pseudodicrogaster japonica* (Machida, 1996) and considered the genus to be within the Haploporinae Nicoll, 1914.

In the phylogenetic analysis by Blasco-Costa et al. (2009a), *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 was resolved outside of the Haploporinae, leading them to erect the Forticulcitinae Blasco-Costa, Balbuena, Kostadinova, et Olson, 2009. In Chapter III, I described two additional species of *Forticulcita*, erected a new genus, and provided an amended diagnosis for the Forticulcitinae. I consider the principle morphological feature uniting the forticulcitines to be a vitellarium that is a single spherical to irregular mass, which *P. japonica* does not possess. Thus, I agreed with Blasco-Costa et al (2009c) in considering that *Pseudodicrogaster* was better attributed to the Haploporinae than the Forticulcitinae; however, I noted that molecular data were lacking.

Andres et al. (2014a) erected *Litosaccus* Andres, Pulis, Cribb et Overstreet, 2014 for *Litosaccus brisbanensis* (Martin, 1973) and used Bayesian inference (BI) analysis of partial 28S rDNA sequences to show that *L. brisbanensis* is a haploporine, the first confirmed haploporine member outside of the Mediterranean Sea. However, Pulis (2014) used BI analysis of sequences of the same gene region to demonstrate that *Unisaccus* Martin, 1974, previously

considered a haploporine (Overstreet and Curran 2005a, Blasco-Costa et al. 2009c) is actually a derived waretrematine. His finding further complicates the morphological basis of the haploporids (see Chapter I), and left *Pseudodicrogaster* and *Rondotrema* Thatcher, 1999 as the only two haploporine genera (sensu Overstreet and Curran 2005a, Blasco-Costa et al. 2009a) without molecular data. Thus, I describe three species of *Pseudodicrogaster* from Australia and use a BI of partial 28S rDNA sequences to test their phylogenetic affinity. The phylogenetic affinity of *Rondotrema microvitellarum* Thatcher, 1999 is also discussed.

Materials and Methods

During February and March of 2010, specimens of the squaretail mullet, *Ellochelon vaigiensis* (Quoy et Gaimard), were collected using a cast-net from locations in Western Australia, Northern Territory, and Queensland, Australia, and examined for trematodes. Haploporids were isolated following the method of Cribb and Bray (2010) for gastrointestinal species but with skipping the initial examination under a dissecting microscope because of the large volume of intestinal contents. The worms were rinsed and cleaned in a container with saline and examined briefly; then, most of the saline was decanted, the worms were killed by pouring hot (not boiling) water over them, and they were fixed in 70% ethanol. Worms were stained in Mayer's haematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted permanently in Dammar gum. Measurements were made using a compound microscope equipped with a differential interference contrast, a Canon EOS Rebel T1i

camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres and data for the holotype are followed by the range of data for the other specimens in parenthesis. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis and Overstreet (2013). Museum abbreviations are as follows: MNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia; QM, Queensland Museum, Brisbane, Queensland, Australia; USNM, Smithsonian National Museum of Natural History, Washington, D.C ., U.S.A.; and WAM, Western Australian Museum, Perth, Western Australia, Australia. Representative specimens will be submitted to museums before the chapter is submitted for publication, thus collection numbers for new material are listed as to be determined (TBD).

Genomic DNA was isolated from specimens either fixed in cool 95% ethanol or heat killed worms in 70% ethanol using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,500-3,000 base pairs (bp) long, comprising the 3' end of the 18S nuclear rRNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S rRNA gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction (PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-

CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3') and the internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences from the species were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Sequences obtained from GenBank are as follows: *Atractotrema sigani* Durio et Manter,1969 (AY222267) (Olson et al. 2003), *Cadenatella isuzumi* Machida, 1993 (FJ788497) (Bray et al. 2009), *Cadenatella pacifica* (Yamaguti, 1970) (FJ788498) (Bray et al. 2009), *Capitimitta costata* Pulis et Overstreet, 2013 (KC206497) (Pulis and Overstreet 2013), *Capitimitta darwinensis* Pulis et Overstreet, 2013 (KC206498) (Pulis and Overstreet 2013), *Capitimitta* sp. (KC206499) of Pulis and Overstreet (2013), *D. contracta* (FJ211262) (Blasco-Costa et al. 2009a), *Dicrogaster perpusilla* Looss, 1902 (FJ211238) (Blasco-Costa et al. 2009a), *F. gibsoni* (FJ211239) (Blasco-Costa et al. 2009a), *Hapladena nasonis* Yamaguti, 1970 (AY222265) (Olson et al. 2003), *Haploporus benedeni* Looss, 1902 (FJ211237) (Blasco-Costa et al. 2009a), *Intromugil alachuaensis* Pulis, Fayton, Curran, et Overstreet, 2013 (KC430095) (Pulis et al.

2013), *Intromugil mugilicolus* (Shireman, 1964) (KC430096) (Pulis et al. 2013), *Lecithobotrys putrescens* Looss, 1902 (FJ211236) (Blasco-Costa et al. 2009a), *Litosaccus brisbanensis* (KM253765) (Andres et al. 2014a), *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko, et Nikitenko, 2014 (HF548461) (Besprozvannykh et al. 2014), *Parasaccocoelium mugili* Zhukov, 1971 (HF548468) (Besprozvannykh et al. 2014), *Parasaccocoelium polyovum* (HF548474) (Besprozvannykh et al. 2014), *Pseudomegasolena ishigakiense* Machida et Kamiya, 1976 (AY222266) (Olson et al. 2003), *Saccocoelioides* sp. of Curran et al. (2006) (EF032696), *Saccocoelium brayi* Blasco-Costa, Balbuena, Raga, Kostadinova, et Olson, 2010 (FJ211234) (Blasco-Costa et al. 2010), *Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009 (FJ211233) (Blasco-Costa et al. 2009a), *Saccocoelium obesum* Looss, 1902 (FJ211260) (Blasco-Costa et al. 2009a), *Saccocoelium tensum* Looss, 1902 (FJ211258) (Blasco-Costa et al. 2009a), and *Spiritestis herveyensis* Pulis et Overstreet, 2013 (KC206500) (Pulis and Overstreet 2013). Sequences of *Forticulcita* sp. n. 1 (SSC23), *Forticulcita* sp. n. 2 (SJ3-1), and Gen. n. 1 *fastigata* (MJA281) from Chapter III and sequences of *Carassotrema estuarinum* Tang et Lin, 1979 (EP198), Park *Malabarotrema lobolecithum* (Martin, 1973) (EP568), *Malabarotrema megaorchis* Liu et Yang, 2002 (EP644), *Malabarotrema* sp. 1 (EP148), *Unisaccoides vitellosus* Martin, 1973 (EP379), *Unisaccoides* sp. 1 (EP077), *Unisaccus brisbanensis* Martin, 1973 (EP376), *Unisaccus lizae* (Liu, 2002) (EP640), *Unisaccus* sp. 1 (EP227), and *Unisaccus* sp. 2 (EP591) from Pulis (2014) are also used. The sequences were aligned

using MAFFT version 6.611b (Katoh et al. 2005) with 1,000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). The resulting alignment utilized two atractotrematids, two species of *Cadenatella*, and 38 haploporids with the atractotrematid *A. sigani* as the outgroup based on its phylogenetic position relative to the Haploporoidea (Andres et al., 2014). Phylogenetic analysis of the data was performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba et al. 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The following model parameters were used in MrBayes: nst = 6, rates = invgamma, ngen = 1,000,000 and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al., 2001) with all other settings left as default.

Results

Pseudodicrogaster sp. n. 1 Figures 4.1A, 4.2A.

Description (measurements based on 11 gravid wholemounts): Body elongate, cylindrical, 942 (836-1,121) long, 144 (118-187) wide at first 1/3 of body length (BL) representing 15% (11-16%) of BL. Tegumental spines 1-2 (1-2)

long, becoming sparse in last third of BL. Eyespot pigment diffuse in forebody. Forebody 280 (246-310) long representing 30% (21-32%) of BL. Hindbody 608 (519-887) long representing 65% (62-75%). Oral sucker subglobular, subterminal 58 (54-68) long, 60 (59-79) wide. Ventral sucker subglobular, 54 (49-62) long, 47 (46-58) wide. Ratio of oral sucker to ventral sucker widths 1: 0.8 (1: 0.7-0.9). Prepharynx 38 (39-58) long. Pharynx subglobular 51 (43-60) long, 58 (41-66) wide. Ratio of oral sucker width to pharynx width 1: 1.0 (1: 0.7-1.0). Oesophagus 186 (171-311) long, extending approximately to level of anterior 1/3 to 2/5 of BL. Caeca approximately 3 to 5 times longer than wide, terminating 413 (287-526) from posterior end representing 44% (34-47%) of BL.

Testis single, elongate, slightly diagonal, median, 125 (101-151) long, 63 (64-77) wide, 196 (151-388) from posterior margin of ventral sucker. Posttesticular field representing approximately 32% (28-40%) of BL. External seminal vesicle tubular, 214 (114-229) long, 26 (20-30) wide, sigmoid in some, posterior to ventral sucker. Hermaphroditic sac recurved, 248 (201-357) long representing 26% (21-31%) of BL, 4.6 (3.9-6.1) times longer than ventral sucker, 62 (66-113) wide at widest point, containing sigmoid internal seminal vesicle measuring 192 (165-219) long by 31 (30-55) wide in posterior portion with prostatic bulb 29 (31-60) long by 23 (22-36) wide; with short male duct; with female duct 95 (72-139) long with male and female ducts uniting at approximately midlevel of hermaphroditic sac; hermaphroditic duct sigmoid, with 1-2 turns, muscularised, lined with pads, 235 (203-269) long.Genital atrium 14 (11-17) deep. Genital pore medial, at level of anterior margin of ventral sucker.

Ovary elongate, subglobular, medial, 67 (58-87) long, 54 (39-58) wide, 182 (128-479) from posterior margin of ventral sucker, contiguous with testis, intercaecal. Laurer's canal not observed. Vitellarium coalesced in two subglobular masses, approximately equal in size, connected by swollen vitelline duct, contiguous with to 24 posterior to testis, 53 (48-65) long by 30 (23-37) wide, 330 (211-488) from posterior margin of ventral sucker. Uterus emerging from posterior margin of ovary, winding posteriorly to near posterior margin of body then anteriorly, occupying most of hindbody, proximal portion filled with sperm. Eggs thin-shelled, numerous, with those in distal portion of uterus 64-65 (61-66) long, 24-25 (24-27) wide; eggs of most specimens in distal portion of uterus with developed miracidia containing 2 separate to fused eyespots.

Excretory vesicle I-shaped, 285 (162-494) long representing 30% (20-44%) of BL; pore terminal.

Type and only known host: *Ellochelon vaigiensis* (Quoy et Gaimard), squaretail mullet, Mugilidae.

Type-locality: Ludmilla Creek, Darwin, Northern Territory, Australia (12^{o}24'52"S, 130^{o}50'12"E).

Other localities: Doyle's Boat Ramp, Fannie Bay, Darwin, Northern Territory, (12^{o}26'09"S, 130^{o}49'56"E); Coconut Wells, Broome, Western Australia, (17^{o}49'13"S, 122^{o}12'40"E); Cable Beach, Broome, Western Australia, (17^{o}55'34"S, 122^{o}12'33"E).

Site: Intestine.

Holotype: MNT TBD.

Paratypes: MNT TBD; NMNH TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP251) from 5 identical sequences (2 adult specimens from Ludmilla Creek, Darwin, Northern Territory; 1 adult specimen from Fannie Bay, Darwin, NT; 1 adult specimen from Coconut Wells; 1 adult specimen from Cable Beach).

Remarks. *Pseudodicrogaster* sp. n. 1 can be distinguished from the other Australian species by having a prepharynx length less than or equal to the pharynx length, mature eggs longer than 61 µm rather than less than 58 µm, and an internal seminal vesicle longer than1.5 times the length of the external seminal vesicle rather than the internal seminal vesicle being greater than 1.5 times the length of the external seminal vesicle.

Pseudodicrogaster sp. n. 2 Figures 4.1B, 4.1B.

Description (measurements based on 13 gravid wholemounts): Body elongate, cylindrical, tapering anteriorly, 867 (690-992) long, 111 (109-169) wide at first 1/3 of BL representing 13% (13-20%) of BL. Tegumental spines 1-2 (1-3) long, becoming sparse in last third of BL. Eyespot pigment diffuse in forebody. Forebody 258 (189-277) long representing 30% (22-30%) of BL. Hindbody 563 (457-724) long representing 65% (64-71%). Oral sucker subglobular, subterminal 43 (39-61) long, 52 (40-63) wide. Ventral sucker subglobular, 46 (42-55) long, 41 (39-56) wide. Ratio of oral sucker to ventral sucker width 1: 0.8 (1: 0.7-1.0). Prepharynx 51 (50-63) long. Pharynx subglobular, 41 (29-46) long, 42 (34-52) wide. Ratio of oral sucker width to pharynx widths 1: 0.8 (1: 0.7-1.0).

Oesophagus 283 (156-389) long, extending posteriorly to approximately level of first 1/3 to 1/2 of BL. Caeca approximately 3 to 4 times longer than wide, terminating 284 (253-496) from posterior end representing 33% (35-52%) of BL.

Testis single, elongate, slightly diagonal, median, longer than caeca, 188 (159-203) long, 71 (60-91) wide, 206 (94-216) from posterior margin of ventral sucker. Posttesticular field representing approximately 19% (12-33%) of BL. External seminal vesicle tubular, 114 (76-122) long, 22 (11-30) wide, slightly sigmoid in some, posterior to ventral sucker. Hermaphroditic sac recurved, 211 (214-318) long representing 24% (22-36%) of BL, 4.6 (4.5-7.0) times longer than ventral sucker, 70 (72-97) wide at widest point, containing sigmoid internal seminal vesicle measuring 206 (192-274) long by 27 (26-38) wide in posterior portion with prostatic bulb 30 (24-47) long by 23 wide (20-30); short male duct, female duct 96 (97-135); male and female ducts uniting at approximately midlevel of hermaphroditic sac; hermaphroditic duct slightly sinus to curved, muscularised, lined with pads ,263 (259-293) long. Genital atrium 15 (10-16) deep. Genital pore medial, at level of anterior margin of ventral sucker.

Ovary elongate, subglobular, medial, 67 (60-79) long, 57 (39-60) wide, 102 (37-163) from posterior margin of ventral sucker, 34 (1-15) anterior to or contiguous with testis, intercaecal. Laurer's canal not observed. Vitellarium coalesced in two subglobular masses, approximately equal in size, connected by swollen vitelline duct, contiguous with testis, 52 (38-60) long by 41 (33-44) wide, 147 (113-227) from posterior margin of ventral sucker. Uterus emerging from posterior margin of ovary, winds posteriorly to approximately level of

midhindbody or near posterior margin of body (4 specimens) then anteriorly, proximal portion filled with sperm. Eggs thin-shelled, numerous, with those eggs in distal portion of uterus 52-54 (52-57) long, 18-21 (17-22) wide; eggs of most specimens in distal portion of uterus with developed miracidia containing 2 separate to fused eyespots.

Excretory vesicle I-shaped, 340 (243-471) long representing 39% (25-49%) of BL; pore terminal.

Type and only known host: *Ellochelon vaigiensis* (Quoy et Gaimard), squaretail mullet, Mugilidae.

Type-locality: off Coconut Wells, Broome, Western Australia, Australia (27^{o}19'47"S, 153^{o}5'11"E).

Other localities: Doyle's Boat Ramp, Fannie Bay, Darwin, Northern Territory, (12^{o}26'09"S, 130^{o}49'56"E); Fish Creek, Corio Bay,Yepoon, Queensland (22^{o}57'53"S, 150^{o}46'26"E).

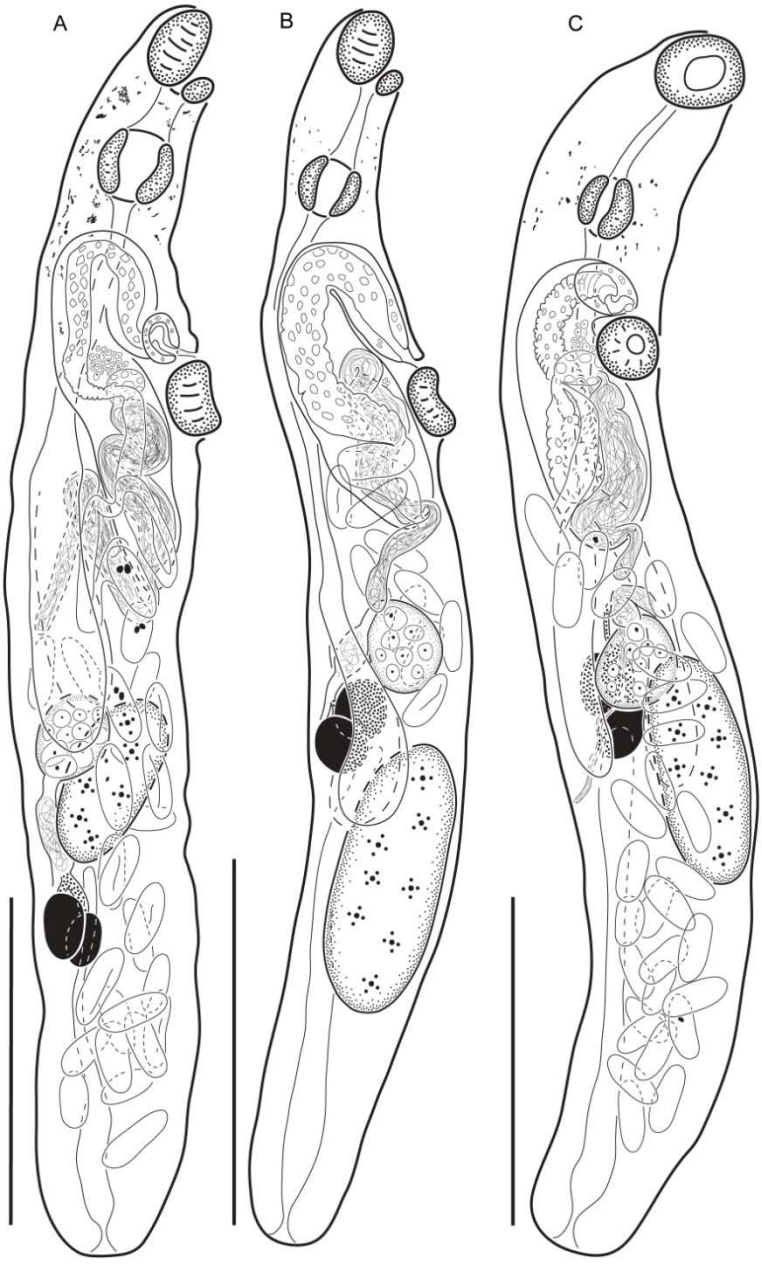
Site: Intestine.

Holotype: WAM TBD.

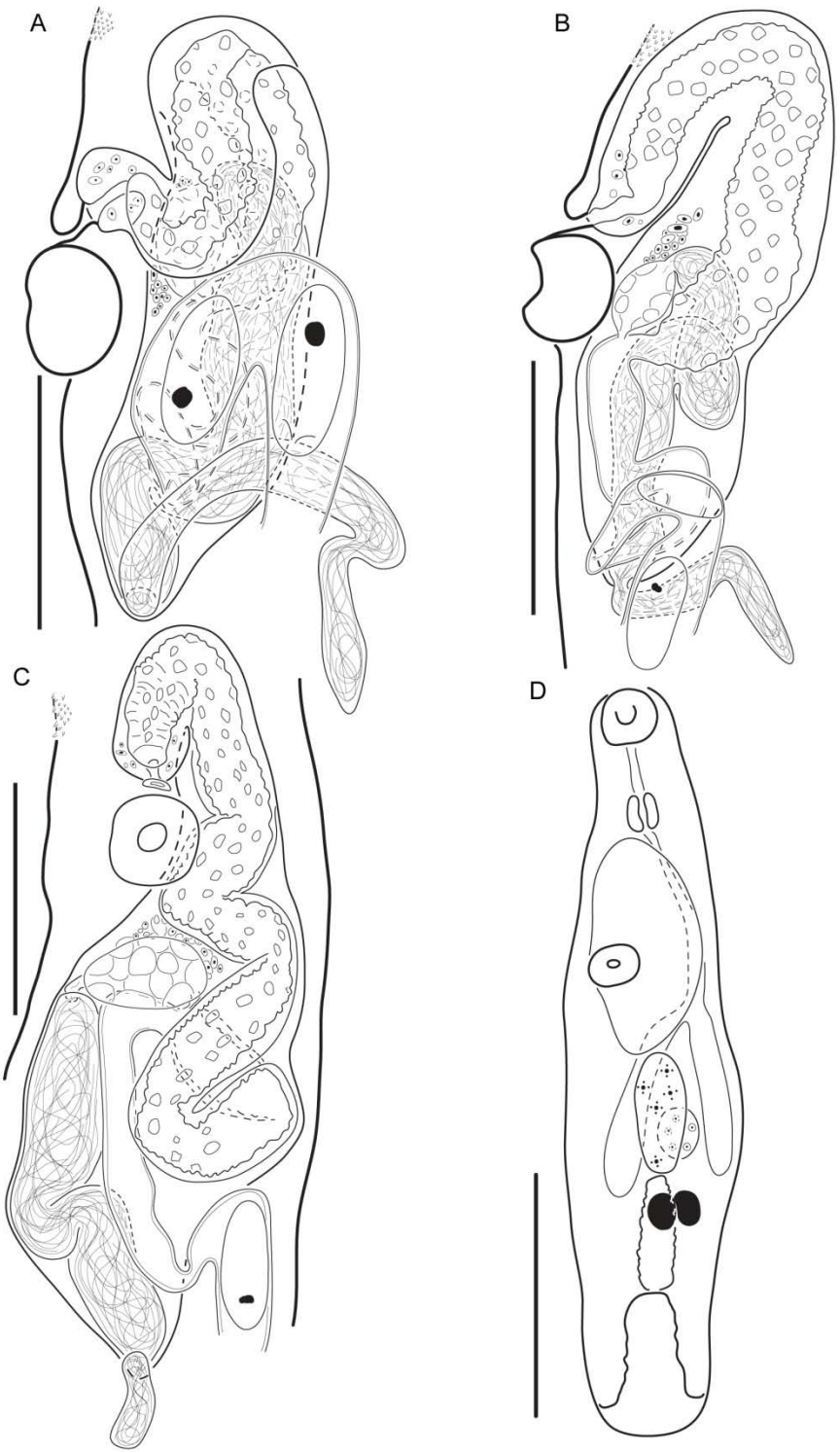
Paratypes: MNT TBD; QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP126) from consensus sequence of 5 specimens. The partial 18S and ITS1 region is from 1 specimen from Coconut Wells, WA; the 5.8S, ITS2, and partial 28S are from 5 identical sequences (4 adult specimens from Coconut Wells, WA; 1 adult specimen from Corio Bay, QLD).

Remarks. *Pseudodicrogaster* sp. n. 2 can be distinguished from the other Australian species by possessing a testis length to caeca length ratio less than 1: 1.



Figures 4.1. *Pseudodicrogaster* sp. n. 1, *Pseudodicrogaster* sp. n. 2, and *Pseudodicrogsater* sp. n. 3. A. *Pseudodicrogaster* sp. n. 1, lateral wholemount. B. *Pseudodicrogaster* sp. 2, lateral wholemount. C. *Pseudodicrogaster* sp. 3, lateral wholemount. Scale-bars A-C, 250 µm.



Figures 4.2. Pseudodicrogaster sp. n. 1, *Pseudodicrogaster* sp. n. 2, and *Pseudodicrogsater* sp. n. 3. A. *Pseudodicrogaster* sp. 1, lateral view of hermaphroditic sac. B. *Pseudodicrogaster* sp. 2, lateral view of hermaphroditic sac. C. *Pseudodicrogaster* sp. 2, ventral view of hermaphroditic sac. D. *Pseudodicrogsater* sp. 3., retracted posterior end, note shift in position of ovary. Scale-bars A-C, 100 µm; D 250 µm.

Pseudodicrogaster sp. n. 3 Figures 4.1C, 4.2C-D.

Description (measurements based on 21 gravid wholemounts): Body elongate, cylindrical, truncated anteriorly, 979 (769-1,277) long, 140 (105-191) wide at first 1/3 of BL representing 14% (11-18%) of BL. Tegumental spines 2-3 (1-3) long, becoming sparse in last third of BL. Eyespot pigment diffuse in forebody. Forebody 249 (213-398) long representing 25% (24-31%) of BL. Hindbody 681 (501-823) long representing 70% (63-71%). Oral sucker subglobular, subterminal 60 (44-67) long, 46 (44-70) wide. Ventral sucker subglobular, 49 (40-57) long, 46 (39-52) wide. Ratio of oral sucker to ventral sucker widths 1: 0.8 (1: 0.7-1.0). Prepharynx 94 (59-96) long. Pharynx subglobular 39 (31-49) long, 36 (24-47) wide. Ratio of oral sucker width to pharynx width 1: 0.6 (1: 0.5-0.8). Oesophagus 221 (171-366) long, extending approximately to level of anterior 1/3 to 2/5 of BL. Caeca approximately 6 to 10 (12 in one specimen) times longer than wide, terminating 362 (236-451) from posterior end representing 37% (31-49%) of BL.

Testis single, elongate, slightly diagonal, sinistral to median, 174 (104-205) long, 76 (58-88) wide, 205 (173-318) from posterior margin of ventral sucker. Posttesticular field representing approximately 30% (17-33%) of BL. External seminal vesicle tubular, 48 (35-114) long, 16 (14-37) wide, posterior to ventral sucker. Hermaphroditic sac, recurved, 231 (212-310) long, 86 (73-131) wide representing 24% (20-35%) of BL, 4.7 (4.5-7.5) times longer than ventral sucker, containing sigmoid internal seminal vesicle 143 (124-211) long by 32 (26-39) widewith prostatic bulb 31 (31-61) long by 25 (21-35) wide; short male

duct; female duct 101 (69-118) long, and hermaphroditic duct sigmoid, with 1-4 turns, muscularised, lined with pads, 322 (273-482) long; male and female ducts uniting at approximately midlevel of hermaphroditic sac; hermaphroditic duct sigmoid, with 1-4 turns, muscularised, lined with pads. Genital atrium 13 (6-14) deep. Genital pore medial, at level of anterior margin of ventral sucker.

Ovary elongate, subglobular, medial, 64 (52-81) long, 53 (46-65) wide, 176 (104-221) from posterior margin of ventral sucker, contiguous with anterior margin of testis to 3-69 anterior to testis, intercaecal. Laurer's canal containing seminal receptacle, opening dorsally, postcaecal. Vitellarium coalesced in two subglobular masses, approximately equal in size, connected by swollen vitelline duct, contiguous with testis, 46 (31-61) long by 29 (21-35) wide, 226 (153-267) from posterior margin of ventral sucker. Uterus emerging from anterior margin of ovary, winding posteriorly to near posterior margin of body then anteriorly, occupies most of hindbody, proximal portion filled with sperm. Eggs thin-shelled, numerous, with those in distal portion of uterus 52-55 (51-56) long, 21-22 (21-24) wide; eggs of most specimens in with developed miracidia containing 2 separate to fused eyespots.

Excretory vesicle I-shaped, 417 (267-518) long representing 43% (33-44%) of BL; pore terminal.

Type and only known host: *Ellochelon vaigiensis* (Quoy et Gaimard), squaretail mullet, Mugilidae.

Type-locality: Withnell Bay, Western Australia, Australia (20^{o}35'3"S, 116^{o}47'20"E).

Other localities: Ludmilla Creek, Darwin, Northern Territory, Australia (12°24'52"S, 130°50'12"E).

Site: Intestine.

Holotype: WAM TBD.

Paratypes: WAM TBD; MNT TBD.

Representative DNA sequences: Partial 18S, 5.8S, ITS2, partial (D1–D3) 28S: GenBank accession no. TBD (MJA588), from 8 identical sequences (7 adult specimens from Withnell Bay, WA; 1 adult Ludmilla Creek, Darwin, NT); ITS1 region GenBank accession no. TBD (EP082; EP588) from 2 identical sequences (2 adult specimens from Withnell Bay, WA), GenBank accession no. TBD (EP083) from 1 sequence (adult specimen from Withnell Bay, WA), and GenBank accession no. TBD (EP222; EP250; MJA885) from 3 sequences (2 adult specimen from Withnell Bay, WA; 1 adult Ludmilla Creek, Darwin, NT[EP250]).

Remarks. *Pseudodicrogaster* sp. n. 3 can be differentiated from the other Australian species of *Pseudodicrogaster* in possessing a prepharynx nearly twice as long as the pharynx, caeca that are 5 to 12 times longer than wide, and a hermaphroditic duct that is more than three times longer than the external seminal vesicle (rather than 1-2 times longer in *Pseudodicrogaster* sp. n. 1 and 2-3 times longer in *Pseudodicrogaster* sp. n. 2). Four specimens of *Pseudodicrogaster* sp. n. 3 had the posterior end retracted (Figure 4.2D) and were not used for measurements. Presumably they were fixed with water that was too cool for proper fixation. A representative specimen is illustrated to show

that the position of the ovary in these specimens is contiguous with the posterior half of the testis, not anterior to or contiguous with the anterior margin of the testis as it is in wellfixed specimens. All three Australian species can be distinguished from *P. japonica* in having a hermaphroditic sac comprising approximately 45% of the body length rather than approximately 30% of the body length and a forebody that is approximately 25% or more of the body length rather than less than 20%.

Molecular analysis

The DNA sequence fragment for *Pseudodicrogaster* sp. n. 1 encompasses 38 bp in the 3' end of the 18S gene, 780 bp in the ITS1, 157 bp in the 5.8S, 272 bp in the ITS2, and 1,396 bp of the 5' end of the 28S gene. No intraspecific variation was observed in the sequences obtained from the five specimens sequenced (3 from the greater Darwin, NT, area and 2 from the greater Broome, WA, area). The DNA sequence fragment for *Pseudodicrogaster* sp. n. 2 encompasses 38 bp in the 3' end of the 18S gene, 1,096 bp in the ITS1, 157 bp in the 5.8S, 270 bp in the ITS2, and 1,396 bp of the 5' end of the 28S gene. No intraspecific variation was observed in the five specimens sequenced (4 from Coconut Wells, WA, and 1 from Corio bay, QLD). The DNA sequence fragment for *Pseudodicrogaster* sp. n. 3 encompasses the 38 bp in the 3' end of the 18S gene; 1,016 bp, 1,095 bp, or 1,174 bp in the ITS1 (see below); 157 bp in the 5.8S; 270 bp in the ITS2; and 1,396 bp of the 5' end of the 28S gene. No intraspecific variation was observed in 5.8S, ITS2, and 28S gene sequences obtained from the eight specimens (7 from Withnell Bay, WA, and 1 from Darwin,

NT). The ITS1 sequences of the three species of *Pseudodicrogaster* have a 79 bp indel and repeat region that begins at position 166 and ends at position 718 on the 5' (Figure 4.3). No intraspecific variation was observed in the 5', 'pre-repeat end' or in the 3', 'post-repeat' end (terminology sensu van Herwerden et al. 1999) of ITS1 sequences.

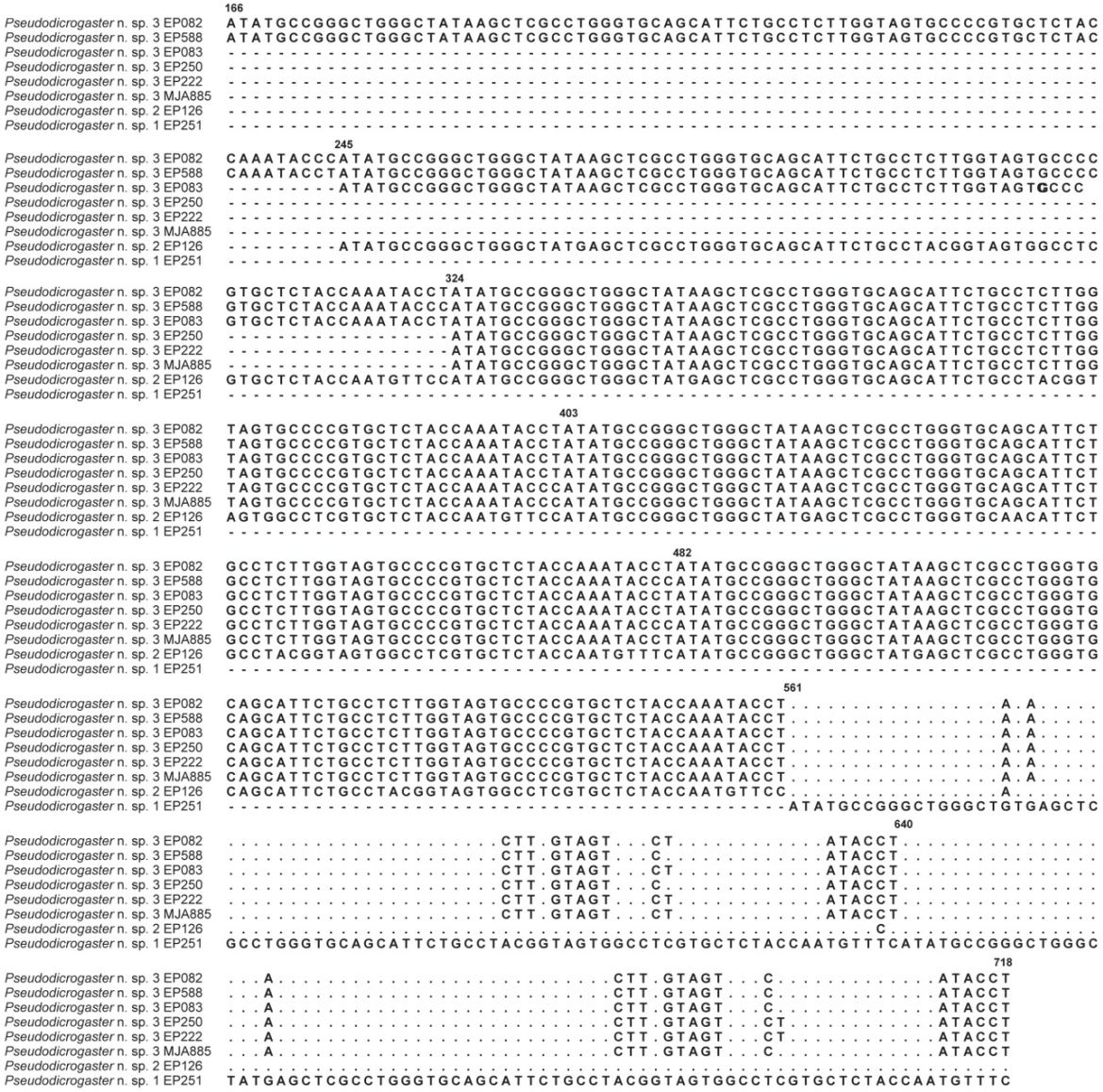


Figure 4.3. Alignment of the variable repeat region of ITS1 sequences obtained from the three species of *Pseudodicrogaster*. Note the variability in the number of repeats in *Pseudodicrogaster* sp. 3. Numbers above sequences correspond with bp position of relative to the longest sequence and appear at the beginning of each repeat.

The sequences of the partial 18S were identical for all three species. The sequences of the 5.8S region were identical for *Pseudodicrogaster* sp. n. 1 and *Pseudodicrogaster* sp. n. 2 and differed from that of *Pseudodicrogaster* sp. n. 3 by one bp (0.6%). Pairwise comparison of ITS1 sequence data excluding the repeat region is found in Table 4.1. Pairwise comparison of the ITS2 and partial 28S sequence data of the three species of *Pseudodicrogaster* are found in Table 4.2.

Table 4.1

Pairwise comparisons, excluding the repeat region and gaps, of percent nucleotide similarity and number of base pair differences (in parentheses) of the ITS1sequences of the three species of Pseudodicrogaster.

	Pseudodicrogaster sp. n. 1	*Pseudodicrogaster* sp. n. 2	*Pseudodicrogaster* sp. n. 3
Pseudodicrogaster sp. n. 1	-	-	-
Pseudodicrogaster sp. n. 2	98.4 (10)	-	-
Pseudodicrogaster sp. n. 3	91.5 (53)	91.1 (55)	-

Table 4.2

Pairwise comparisons (excluding gaps) of percent nucleotide similarity and number of base pair differences (in parentheses) of the ITS-2 (below the diagonal) and 28S (above the diagonal) sequences of the three species of Pseudodicrogaster.

	Pseudodicrogaster sp. n. 1	*Pseudodicrogaster* sp. n. 2	*Pseudodicrogaster* sp. n. 3
Pseudodicrogaster sp. n. 1	-	99.6 (6)	97.4 (36)
Pseudodicrogaster sp. n. 2	98.1 (5)	-	97.3 (38)
Pseudodicrogaster sp. n. 3	93.3 (18)	93.7 (17)	-

The alignment of partial 28S rDNA sequences of the three species of *Pseudodicrogaster* and related species from GenBank was 1,127 characters long with 626 conserved sites, 501 variable sites, and 387 informative sites. The BI analysis (Figure 4.4) of those sequences incorporated *A. sigani* as the outgroup, *P*. *ishigaki,* and an ingroup of 39 haploporids. The ingroup of haploporids form a

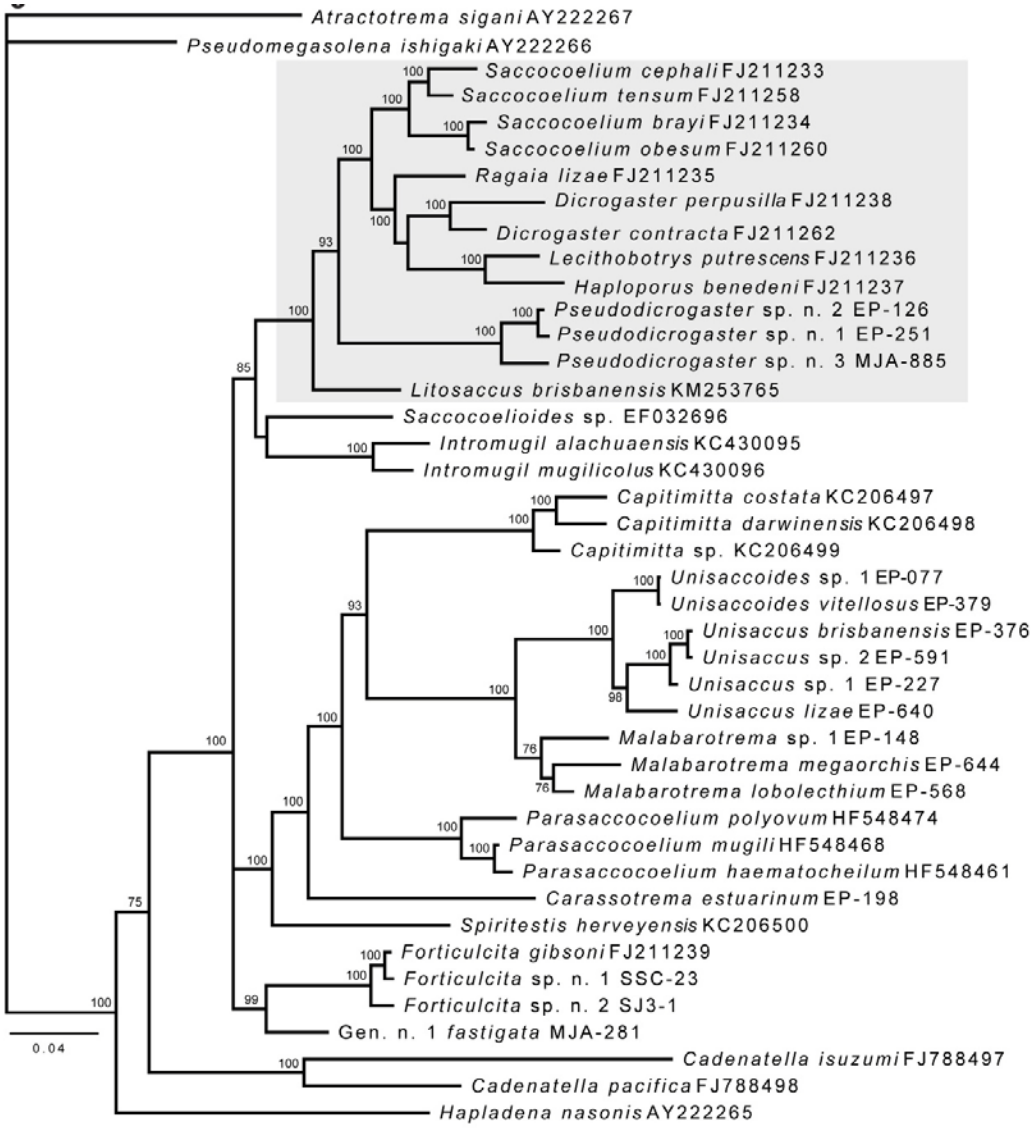


Figure 4.4. Phylogenetic relationships among members of the Haploporidae resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ; 1,000,000 generations and a sample frequency of 100) revealing the three species of *Pseudodicrogaster* as haploporines (shaded rectangle). Support values of <75% not shown.

monophyletic clade. *Hapladena nasonis* was resolved as the basal to the rest of the haploporoids, with species of *Cadenatella* as the sister to the polytomy containing the rest of the haploporids. The polytomy consisted of three major clades: 1) the Forticulcitinae, 2) the Waretrematinae, and 3) *Saccocoiloides* sp. and *Intromugil* spp. + the Haploporinae. *Litosaccus brisbanensis* was resolved as the basal haploporine member; with the three species of *Pseudodicrogaster* as the sister group to the Mediterranean Sea haploporines.

Discussion

The three new Australian species of *Pseudodicrogaster* can be distinguished from *P. japonica* in having a hermaphroditic sac comprising approximately 45% rather than approximately 30% of the body length and a forebody that is approximately 25% or more rather than less than 20% of the body length. *Pseudodicrogaster japonica* also has an internal seminal vesicle that is longer relative to the hermaphroditic duct than it is in *Pseudodicrogaster* sp. n. 1 and *Pseudodicrogaster* sp. n. 2, but similar to that of *Pseudodicrogaster* sp. n. 3. The three new species fit the generic diagnosis by Blasco-Costa et al. (2009c) in having an elongated internal and external seminal vesicle, well-defined pads lining the hermaphroditic duct, and a large hermaphroditic sac in both absolute measurements and relative to body length. Machida's (1996) specimens were fixed under pressure; therefore, the inverse pyriform body, greater body width, the transversely elongate ventral sucker, and the well-defined 'dumbbell'-shaped vitellarium exhibited by his specimens are likely artifacts of fixation technique. The vitellarium in my specimens are two adjacent compact

masses, connected by a swollen vitelline duct, similar in appearance to those of *Dicrogaster* spp. The description of these three Australian species necessitates an emendation to the generic diagnosis by Blasco-Costa et al. (2009c) to include body obovate to cylindrical; ventral sucker subglobular; caeca terminate at approximately level of midbody to final third of body; external seminal vesicle usually smaller than internal seminal vesicle; genital atrium present; eggs numerous or not; vitellarium as two adjacent compact masses, with swollen vitelline duct uniting the two masses; excretory vesicle I-shaped, short to approximately two-fifths of body length; in Indo-Pacific mugilids.

Machida (1996) described *P. japonica* from *M. cephalus*; however, I did not recover any haploporids from 33 specimens of *M. cephalus* examined in Western Australia (sampling locations extending from Carnarvon, WA, to Broome, WA). In 16 specimens of *M. cephalus* examined from Queensland the only haploporids found were *L. brisbanensis* (Martin, 1974) and *Unisaccus brisbanensis* Martin, 1973. No specimens of *M. cephalus* were examined in Northern Territory. Unfortunately, I did not collect specimens of *M. cephalus* from locations where species of *Pseudodicrogaster* were recovered. Therefore, it is not possible to say that *M. cephalus* is not a host for any of the three Australian species of *Pseudodicrogaster*. The feeding ecology of *M. cephalus* and *E. vaigiensis* is slightly different. *Mugil cephalus* feeds primarily on detritus and microalgae (see Whitfield et al. 2012) but *E. vaigiensis* has been found to feed on detritus and microalgae as well as gastropods, polychaetes, phytoplankton, and zooplankton (see Wijeyaratne and Costa 1990, Hajisamae et al. 2004). The

intestinal contents of *E. vaigiensis* I examined were generally filled with green filamentous algae, and these fish were observed feeding on hard substrate (e.g., boat ramps, bulkheads, rocks) near the water surface, but the intestinal contents of *M. cephalus* examined consisted largely of detritus and sediment. Thus, the two hosts foraging in different habitats could be a reason for the lack of *Pseudodicrogaster* spp. infection in *M. cephalus*.

Blasco-Costa et al. (2010) suggested that the greater than expected number of sympatric species of *Saccocoelium* infecting Mediterranean mugilids likely pointed towards those species radiation with their gastropod first intermediate host. They suggested that speciation within *Saccocoelium* Looss, 1902 could be related to cryptic diversification of the gastropod or localized adaptation, determined by the spatial structure of intermediate hosts with direct development. The three Australian species of *Pseudodicrogaster* all occurred in the same host species and geographically overlapped with each other. All three species were collected in the greater Darwin area and *Pseudodicrogaster* sp. n. 1 and *Pseudodicrogaster* sp. n. 2 were both collected off Broome. Hosts ranged in size from 5 cm to 35 cm, but with no distribution that would suggest a parasite species shift with host age (length). Unfortunately, the lifecycle of any species of *Pseudodicrogaster* is unknown. I suspect the first intermediate hosts for the Australian species of *Pseudodicorgaster* live in association with the green filamentous algae that was the source of food eaten by the infected fish hosts. Thus, future studies should examine the gastropods in these environments to provide insight on whether or not the geographical overlap of species is related to

movement of the final host or to the occurrence of the appropriate intermediate hosts across the geographic area.

The length of ITS1 sequences of *Pseudodicrogaster* sp. n. 3 was 1,016 bp, 1,095 bp, or 1,174 bp depending on the number of 79 bp repeats. Variable numbers of repetitive indels in the ITS1 have been reported both within species and within individuals of other trematode species (e.g., van Herwerden et al. 1999, Nolan and Cribb 2005, Warberg et al. 2005,Heneber and Literák 2013). Therefore, the ITS1 is does not seem to be a reliable molecular marker in delineation of haploporid species.

My BI hypothesis revealed that the three species of *Pseudodicrogaster* formed a monophyletic clade within the Haploporinae and sister to the Mediterranean species. The topology was similar to the most recent hypotheses (Bray et al. 2014, Andres et al. 2014a, Chapter III), with *Hapladena nasonis* as the basal clade and species of *Cadenatella* as the sister group to the polytomy leading to the rest of the tested haploporids. *Unisaccus* was well resolved within the waretrematinae, with *S*. *herveyensis* as the sister to the rest of the waretrematines.

Andres et al. (2014a) stated that molecular data for members of *Pseudodicrogaster*, *Rondotrema*, and *Unisaccus* were still needed to test the morphological framework of the subfamily by Overstreet and Curran (2005a) and Blasco-Costa et al. (2009c). My BI hypothesis confirms that *Pseudodicrogaster* is a haploporine genus, and Pulis (2014) found that *Unisaccus* was misplaced. The subfamilial affinity of *Rondotrema* is still uncertain; however, I suggest

Rondotrema is better allocated to the Chalcinotrematinae Overstreet et Curran, 2005.

Thatcher (1999) described *Rondotrema microvitellarum* Thatcher, 1999 from the characiforme *Hemiodus microlepis* Kner from the Guaporé River, Brazil. I consider the characiforme host and South American, freshwater locality to indicate that the monotypic genus is likely more closely affiliated with the Chalcinotrematinae. Overstreet and Curran (2005a) considered members of the Chalcinotrematinae to possess an extensive uterus (occupying much of the hindbody and often extending into the forebody); either irregularly elongate vitelline follicles that surround the testis or follicles that are irregularly dispersed in hindbody; and infecting estuarine and freshwater fishes in the New World and Africa. With the recent realization that the morphological features of the haploporids are more plastic than previously thought (Blasco-Costa et al. 2009a, Blasco-Costa et al. 2010, Pulis and Overstreet 2013, Bray et al. 2014, Pulis 2014, Chapter III), the data on host and geographic locality may be more reliable. Pulis (2014) used BI analysis of 28S rDNA sequences to show that *Unisaccus* is better allocated to the Waretrematinae. His transfer of *Unisaccus* to the waretrematinae leaves only the Megasoleninae Manter, 1935 (which contains members that mature in marine, reef associated fishes) without a representative that exhibits a reduced vitellarium and mature eggs containing miricidia with eyespots. Pearson (1968) considered the combination of a reduced vitellarium and fully embryonated eggs to be a significant life-history trait for miricidia to find a gastropod intermediate host in intertidal environments, as miricida that develop

within eggs in the intertidal zone would be prone to desiccation. The convergent evolution exhibited by some members of the Forticulcitinae, Haploporinae, and Waretrematinae supports this hypothesis. Presumably, *H. microlepis* is not subject to the intertidal environment; however, *H*. *microlepis* is an abundant member of floodplain-lakes and flooded forests of the Amazon (e.g., Lin and Caramaschi 2005, Granado-Lorencio et al. 2007). Thus, miracidia of *R. microvitellarium* are potentially subject to an ephemeral habitat, with similar risks for desiccation as the intertidal environment. I examined seven paratypes of *R. microvitellarium*, all of which seem to have been fixed under pressure (Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil INPA 376 a-g). *Rondotrema microvitellarium* possesses vitelline follicles that are on opposite sides of the testis, and two specimens have the uterus extending into the forebody. Both of these characteristics fit with the chalcinotrematines. Thus, I transfer *Rondotrema* to the Chalcinotrematinae.

With the verification that *Pseudodicrogaster* is a haploporine and my proposal to transfer *Rondotrema* to the Chalcinotrematinae, the Haploporinae contains seven genera, all of which have molecular data coupled with morphological data. However, Blasco-Costa et al. (2009b) considered five Indo-Pacific species of *Haploporus* Looss, 1902 as *incertae sedis* with respect to their generic affiliation but maintained them in the Haploporinae. Additionally, Blasco-Costa et al. (2009e) transferred *Saccocoelium megasacculum* Liu, Wang, Peng, Yu, et Yang, 2006 to *Eliptobursa* Wu, Lu, et Zhu, 1996, a genus that previously was considered in the Monorchiidae Odhner, 1911 (see Madhavi 2008). Neither

Madhavi (2008) nor Blasco-Costa et al. (2009e) proposed a subfamilial association for *Eliptobursa*, although presumably the members of the genus would best accommodated by the Haploporinae. Therefore, additional attention is required to generate a clearer picture of the Haploporiane.

CHAPTER V

A NEW GENUS OF INDO-WEST PACIFIC HAPLOPORINE (TREMATODA: HAPLOPORIDAE NICOLL, 1914)

Abstract

Gen. n. 2 is erected for four new species from Australia and five new combinations are proposed. Gen. n. 2 is morphologically most similar to the haploporine genera *Haploporus* Looss, 1902, *Dicrogaster* Looss, 1902, and *Saccocoelium* Looss, 1902, but can be differentiated from all three in possessing a saccate, I-shaped excretory vesicle, often containing a concretion. Gen. n. 2 can be further differentiated from *Haploporus* and *Dicrogaster* by having an oesophagus that is more than twice the length of the pharynx. I describe Gen. n. 2 sp. n. 1 the type species genus because it is the first to be coupled with molecular data. *Saccocoelium megasacculum* Liu, Wang, Peng, Yu, et Yang, 2004, *Haploporus sac*, and X species are described. My Bayesian inference analysis revealed the members of Gen. n. 2 in an unresolved monophyletic clade as the sister group to the rest of the haploporines. *Elliptobursa* Wu, Lu, et Zhu, 1996 and *Allomonorchis* Lu, Wu, et Chen, 1999 are considered *incerte sedis*. A key to the Gen. n. 2 is presented.

Introduction

Haploporus Looss, 1902 was erected for *Haploporus benedeni* (Stossich, 1887) and *Haploporus lateralis* Looss, 1902, both from Mediterranean mugilids. Prior to Overstreet and Curran's (2005a) review of the Haploporidae Nicoll, 1914, eight additional *Haploporus* spp. were reported; *Haploporus longicollum*

Vlassenko, 1931 (now considered a junior synonym of *Saccocoelium obesum* Looss, 1902 [Overstreet and Curran 2005a, Blasco-Costa et al. 2009b) was described from the Mediterranean Sea; *Haploporus lossii* Al-Bassel, 1990 (considered a *nomen nudum* by Blasco-Costa et al. [2009b]) from a freshwater lake in Egypt; and *Haploporus indicus* Rekharani et Madhavi, 1985, *Haploporus magnisaccus* Machida, 1996, *Haploporus mugilis* Liu et Yang, 2002, *Haploporus musculosaccus* Machida, 2003, *Haploporus pseudindicus* Rekharani et Madhavi, 1985, and *Haploporus spinosus* Machida, 1996 were all described from the Indo-West Pacific. Manter (1963) erected *Neohaploporus* Manter, 1963 for *Neohaploporus pacificus* Manter, 1963, a species close to *Haploporus* but differing morphologically in possessing a longer, more elongate body; longer caeca; lymphatic vessels; and infecting a scatophagid rather than mugilid. Overstreet and Curran (2005a) interpreted the lymphatic vessels to be gland cells associated with the oral sucker and they considered the different host association not to be an ‘unnatural host grouping’ because of the similar feeding ecologies of the two host families. Thus, they determined the differences to be of specific value and considered *Neohaploporus* to be a junior synonym of *Haploporus*, bringing the total number of Indo-West Pacific *Haploporus* spp. to seven.

Blasco-Costa et al. (2009b) revised *Haploporus* and *Lecithobotrys* Looss, 1902 and in doing so considered *H. lateralis* to be a junior synonym of *H. bendeni*. They also suggested that the Indo-Pacific forms possessed characters that were not in common with Overstreet and Curran’s (2005a) generic diagnosis

of *Haploporus*; namely, an armed hermaphroditic duct (in most members), a long oesophagus, a transversely elongate oral sucker, an elongate testis, and a variable genital atrium. Blasco-Costa et al. (2009b) further suggested that the Indo-West Pacific forms, with the exception of *H. pacificus*, may form a monophyletic group, which they referred to as "species from *Valamugil* spp." (118), outside of *Haploporus*. They retained *H*. *indicus*, *H*. *magnisaccus*, *H*. *mugilis*, and *H. musculosaccus*, and *H. spinosus* in the Haploporinae Nicoll, 1914, but as *incertae sedis* with respect to their generic affiliation and considered *H. musculosaccus*, *H*. *pacificus*, and *H*. *pseudindicus* to be *species inquirendae*.

Madhavi (2008) provided a key to the Monorchiidae Odhner, 1911 and considered *Elliptobursa* Wu, Lu, et Zhu, 1996 and *Allomonorchis* Lu, Wu, et Chen, 1999 to be better allocated to the Haploporidae “as evidenced by the presence of a single testis, a long external seminal vesicle, a well-developed prostatic complex, and a long hermaphroditic duct wrongly interpreted as a cirrus” (146), despite the distal portion of the uterus described and illustrated as separate from the cirrus sac. Liu et al. (2004) described *Saccocoelium megasacculum* Liu, Wang, Peng, Yu, et Yang, 2004 from *Liza affinis* (Günther) (as *Liza carinatus* [Cuvier et Valenciennes]) in the Taiwan Strait. Blasco-Costa et al. (2009e) revised *Saccocoelium* Looss, 1902 and transferred *S. megasaculum* to *Elliptobursa* as *Elliptobursa megasaculum* (Liu, Wang, Peng, Yu, et Yang, 2004). Although they did not suggest a subfamilial association, presumably they considered it close to members of the Haploporinae. Blasco-Coasta et al.

(2009b) suggested that *Elliptobursa* may also be a possible repository for the '*Valamugil* spp.' species of *Haploporus*.

Blasco-Costa et al. (2009a) provided a molecular hypothesis to the Haploporinae and in doing so provided molecular data for the type species of *Haploporus*, *H. benedeni*. Their analysis, and recent analyses (Blasco-Costa et al. 2010, Pulis and Overstreet 2013, Bray et al. 2014, Andres et al. 2014a, Pulis 2014, Chapter III), demonstrated that some of the morphological characters that have previously been used to delineate taxa are homoplastic. Therefore, the purpose of this study is to determine the phylogenetic affinity of some Indo-West Pacific haploporines.

Materials and Methods

During February and March 2010 haploporids were collected from mullet species in three genera; *Chelon* Artedi, *Moolgarda* Whitley, and *Valamugil* Smith by cast-net from locations in Western Australia, Northern Territory, and Queensland, Australia. Chinese specimens were obtained from (?) *Chelon subviridis* (Valenciennes) purchased live at a fish market near Daya Bay, Guangdong Province, China, by Eric Pulis in March 2009. Vietnamese specimens were obtained from (?) *C. subviridis* purchased from a fish market in Nha Trang, Vietnam, by Robin Overstreet and Stephen Bullard in January 2007. The hosts obtained from fish markets were assumed to be from the nearby water bodies. Specific fish names follow those given by FishBase (Froese and Pauly 2014). Haploporids were isolated following the method of Cribb and Bray (2010) for gastrointestinal species but skipping the initial examination under a dissecting

microscope because of the large volume of intestinal contents. The worms were rinsed and cleaned in a container with saline and examined briefly; then, most of the saline was decanted, the worms were killed by pouring hot (not boiling) water over them, and they were fixed in 70% ethanol or 10% buffered formalin (Vietnam samples only). Worms were stained in Mayer's haematoxylin, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted permanently in Dammar gum. Measurements were made using a compound microscope equipped with a differential interference contrast, a Canon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres; for descriptions of new species, data for the type specimen are followed by the range of data for the other specimens in parenthesis, and for reports of other species, supplemental data are provided. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis and Overstreet (2013). Additionally, I consider the genital atrium to be the cavity through which an everted hermaphroditic duct passes that, although muscular, is not strongly muscular. I consider the strongly muscular portion of the haploporid terminal genetalia that surrounds the proximal portion of the genital atrium an extension of the distal portion end of the hermaphroditic sac and not a muscular genital atrium as it has been referred to by previous authors. Museum abbreviations are as follows: BMNH, British Museum of Natural History London, England; FJXM, Parasitology Research Laboratory, Xiamen, University, People's Republic of China; MNT, Museum and Art Gallery of the Northern Territory, Darwin, Australia; QM, Queensland Museum, Brisbane, Queensland, Australia;

USNM, Smithsonian National Museum of Natural History, Washington, DC, USA; USNPC, United States National Parasite Collection (previously in Beltsville, Maryland, USA.), and WAM, Western Australian Museum, Perth, Western Australia, Australia. Representative specimens will be submitted to museums before the chapter is submitted for publication, thus collection numbers for new material are listed as to be determined (TBD).

Genomic DNA was isolated from specimens either fixed in cool 95% ethanol or heat killed worms in 70% ethanol using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,500 base pairs (bp) long, comprising the 3' end of the 18S nuclear rRNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S rRNA gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction (PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3') and the internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were

excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences from the species were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Sequences obtained from GenBank are as follows: *Atractotrema sigani* Durio et Manter,1969 (AY222267) (Olson et al. 2003), *Cadenatella isuzumi* Machida, 1993 (FJ788497) (Bray et al. 2009), *Cadenatella pacifica* (Yamaguti, 1970) (FJ788498) (Bray et al. 2009), *Capitimitta costata* Pulis et Overstreet, 2013 (KC206497) (Pulis and Overstreet 2013), *Capitimitta darwinensis* Pulis et Overstreet, 2013 (KC206498) (Pulis and Overstreet 2013), *Capitimitta* sp. (KC206499) of Pulis and Overstreet (2013), *Dicrogaster contracta* Looss, 1902 (FJ211262) (Blasco-Costa et al. 2009a), *Dicrogaster perpusilla* Looss, 1902 (FJ211238) (Blasco-Costa et al. 2009a), *F. gibsoni* (FJ211239) (Blasco-Costa et al. 2009a), *Hapladena nasonis* Yamaguti, 1970 (AY222265) (Olson et al. 2003), *H*. *benedeni* (FJ211237) (Blasco-Costa et al. 2009a), *Intromugil alachuaensis* Pulis, Fayton, Curran, et Overstreet, 2013 (KC430095) (Pulis et al. 2013), *Intromugil mugilicolus* (Shireman, 1964) (KC430096) (Pulis et al. 2013), *Lecithobotrys putrescens* Looss, 1902 (FJ211236) (Blasco-Costa et al. 2009a), *Litosaccus brisbanensis* (KM253765) (Andres et al. 2014a), *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko, et Nikitenko, 2014 (HF548461) (Besprozvannykh et al. 2014), *Parasaccocoelium mugili* Zhukov,

1971 (HF548468) (Besprozvannykh et al. 2014), *Parasaccocoelium polyovum* (HF548474) (Besprozvannykh et al. 2014), *Pseudomegasolena ishigakiense* Machida et Kamiya, 1976 (AY222266) (Olson et al. 2003), *Saccocoelioides* sp. of Curran et al. (2006) (EF032696), *Saccocoelium brayi* Blasco-Costa, Montero, Balbuena, Raga, Kostadinova, et Olson, 2009 (FJ211234) (Blasco-Costa et al. 2009a), *Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009 (FJ211233) (Blasco-Costa et al. 2009a), *Saccocoelium obesum* Looss, 1902 (FJ211260) (Blasco-Costa et al. 2009a), *Saccocoelium tensum* Looss, 1902 (FJ211258) (Blasco-Costa et al. 2009a), and *Spiritestis herveyensis* Pulis et Overstreet, 2013 (KC206500) (Pulis and Overstreet 2013). Sequences of *Forticulcita* sp. n. 1 (SSC23), *Forticulcita* sp. n. 2 (SJ3-1), and Gen. n. 1 *fastigata* (MJA281) from Chapter III; *Pseudodicrogaster* sp. n. 1, *Pseudodicrogaster* sp. n. 2, and *Pseudodicrogaster* sp. n. 3 from Chapter IV; and sequences of *Carassotrema estuarinum* Tang et Lin, 1979 (EP198), Park *Malabarotrema lobolecithum* (Martin, 1973) (EP568), *Malabarotrema megaorchis* Liu et Yang, 2002 (EP644), *Malabarotrema* sp. 1 (EP148), *Unisaccoides vitellosus* Martin, 1973 (EP379), *Unisaccoides* sp. 1 (EP077), *Unisaccus brisbanensis* Martin, 1973 (EP376), *Unisaccus lizae* (Liu, 2002) (EP640), *Unisaccus* sp. 1 (EP227), and *Unisaccus* sp. 2 (EP591) from Pulis (2014) are also used. The sequences were aligned using MAFFT version 6.611b (Katoh et al. 2005) with 1,000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was

trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). The resulting alignment utilized two atractotrematids, two species of *Cadenatella*, and 38 haploporids with the atractotrematid *A. sigani* as the outgroup based on its phylogenetic position relative to the Haploporoidea (Andres et al., 2014). Phylogenetic analysis of the data was performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba et al. 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The following model parameters were used in MrBayes: nst = 6, rates = invgamma, ngen = 1,000,000 and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al. 2001) with all other settings left as default.

Results

Gen. n. 2

Diagnosis. Body of adult elongate, fusiform, slightly more than 4× longer than wide. Tegument spinous. Eyespot pigment very diffuse in forebody to apparently absent. Oral sucker subterminal, transversely elongate. Ventral sucker transversely oval, shorter than oral sucker, in first 1/3 of body. Prepharynx short. Pharynx elliptical to globular, smaller than oral sucker, nearly contiguous to contiguous with posterior margin of oral sucker. Oesophagus 2.5 - 9 times longer

than pharynx. Intestinal bifurcation variable, usually posterior to ventral sucker. Caeca two, sac-like to cylindrical, end blindly at approximately midbody. Testis single, elongate, irregular to elliptical, median, postcaecal, located approximately at level of midbody or further posterior. External seminal vesicle saccular to elongated, glandular or not. Hermaphroditic sac elongate fusiform to elliptical, in first 1/4 to first 1/3 of body length, longer than ventral sucker; sac containing swollen internal seminal vesicle, small to large prostatic complex, glandular female duct, and hermaphroditic duct; often with spines of varying sizes; distal portion of hermaphroditic sac muscular, forming a ring around proximal portion of genital atrium. Genital atrium well-developed. Ovary subglobular to globular, medial, pretesticular. Vitellarium 2 compact masses, symmetrical, globular to subglobular; masses united by large duct giving dumbbell shape; posterolateral to ovary. Uterus occupying most of hindbody. Eggs numerous, containing developed miracidia with eyespots. Excretory vesicle I-shaped, sac-like to elongate, often with concreation, terminating in hindbody. In Mugilidae; in Indo-Pacific Region.

Type-species: Gen. n. 2 sp. 1.

Remarks. Gen. n. 2 is morphologically most similar to the haploporine genera *Haploporus* Looss, 1902, *Dicrogaster* Looss, 1902 and *Saccocoelium* Looss, 1902. Gen. n. 2 is differentiated from all three in possessing an I-shaped excretory vesicle that is generally saccate and containing a concretion in some but not all species and some but not all specimens of those species. Gen. n. 2 can be further differentiated from *Haploporus* and *Dicrogaster* by an oesophagus

that is more than twice the length of the pharynx. Gen. n. 2 sp. 1 is chosen as the type species because it is the first to be coupled with molecular data.

Gen. n. 2 sp. n. 1 Figure 5.1A-B.

Description (measurements based on 9 wholemounts): Body elongate, truncated anteriorly, tapering anterior, with slight constriction at first 1/4 of body length (BL), 1,082 (989-1,274) long, 121 (119-138) wide at first 1/3 of BL representing 11% (11-13%) of BL. Tegumental spines 1-2 (1-3) long, covering surface of ventral sucker, becoming less apparent in last 1/3 of BL. Eyespot pigment very diffuse in forebody. Forebody 333 (290-412) long representing 31% (29-31%) of BL. Hindbody 693 (652-801) long representing 64% (63-66%). Oral sucker subglobular, 52 (44-62) long, 68 (55-79) wide. Ventral sucker subglobular, 56 (47-61) long, 60 (48-63) wide. Ratio of oral sucker to ventral sucker widths 1: 0.9 (1: 0.8-0.9). Prepharynx 4 (6-11) long. Pharynx transversely elongate, subglobular, 37 (31-48) long, 27 (19-37) wide. Ratio of oral sucker width to pharynx width 1: 0.4 (1: 0.4-0.5). Oesophagus 317 (310-388) long, extending approximately to level of anterior third of BL. Caeca approximately 8-11 times longer than wide, terminating 385 (334-474) from posterior end representing 36% (34-37%) of BL.

Testis postcaecal, 126 (112-148) long, 66 (61-82) wide, 351 (310-415) from posterior margin of ventral sucker. Posttesticular field representing approximately 20% (19-23 %) of BL. External seminal vesicle saccular, 24 (18-38) long, 17 (16-21) wide, posterior to ventral sucker. Hermaphroditic sac elongate, 142 (133-178) long, 43 (39-56) wide representing 13% (13-14%) of BL,

2.5 (2.6-2.9) times longer than ventral sucker, with distal portion strongly muscular forming ring with spines; spines 14 in number in distal ring, 12-14 long by 3-4 wide; containing terminal genitalia, internal seminal vesicle 83 (73-109) long, 25 (28-48) wide; prostatic bulb small, 12 (11-16) long, 7 (8-10) wide; male duct short; female duct glandular, 88 (81-124) long; hermaphroditic duct 27 (24-36) long, with male and female ducts uniting between midlevel to level of distal 1/3 of hermaphroditic sac, strongly muscular, containing thin spines. Genital atrium 11 (7-19) deep, with proximal portion surrounded by muscular ring of hermaphroditic sac. Genital pore medial, at level of anterior margin of ventral sucker.

Ovary elongate, postacecal, 77 (61-89) long, 53 (49-58) wide, 296 (249-331) from posterior margin of ventral sucker, contiguous with anterior margin of testis to 5-17 anterior to testis, intercaecal. Laurer's canal containing seminal receptacle, opening dorsally, preovarian. Vitelline masses subglobular, approximately equal in size, contiguous with posterior margin of ovary, contiguous with anterior margin of testis, 44 (42-53) long, 31 (26-42) wide, 301 (279-410) from posterior margin of ventral sucker. Uterus emerging from anterior margin of ovary, winding posteriorly to near posterior margin of body then anteriorly, occupying most of hindbody, proximal portion filled with sperm. Eggs thin-shelled, numerous, with those in distal portion of uterus 35-37 (35-39) long by 17 (16-19) wide; with those in distal portion of uterus with developed miracidia with fused eyespots.

Excretory vesicle I-shaped, saccular, 130 (107-174) long representing 12% (11-14%) of BL; pore terminal.

Type and only known host: *Valamugil buchanani* (Bleeker), bluetail mullet, Mugilidae.

Type-locality: Coconut Wells, Broome, Western Australia, Australia (27^{o}19'47"S, 153^{o}5'11"E).

Site: Intestine.

Holotype: WAM TBD.

Paratypes: USNM TBD; QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-303) from two specimens.

Remarks. Gen. n. 2 sp. n. 1 can be differentiated from the rest of the species in Gen. n. 2 based on having a combination of an armed hermaphroditic duct consisting of a ring of medium sized spines around distal portion but lacking spines along the rest of the hermaphroditic duct; a saccate excretory vesicle; a longer (<25% of body length) forebody; and a preovarian Laurer's canal and Laurer’s canal opening.

Gen. n. 2 sp. n. 2 Figure 5.1C-D.

Description (measurements based on 16 wholemounts): Body elongate, truncated anteriorly, tapered posteriorly, 1,365 (1,190-1,548) long, 213 (208-238) wide at first 1/3 of BL representing 16% (15-17%) of BL. Tegument thin. Tegumental spines 1-2 (1-2) long, in longitudinal rows; rows close in forebody, with those up to twice the distance apart in hindbody as in forebody. Eyespot

pigment very diffuse in forebody. Forebody 399 (308-487) long representing 29% (26-31%) of BL. Hindbody 854 (774-929) long representing 63% (60-65%). Oral sucker 98 (86-108) long, 126 (111-134) wide. Ventral sucker cup shaped, anteriorly facing, 112 (107-136) long, 113 (106-141) wide. Ratio of oral sucker to ventral sucker widths 1: 0.9 (1: 0.9-1.1). Prepharynx 8 (7-13) long. Pharynx elongate, 63 (51-78) long, 49 (38-56) wide. Ratio of oral sucker width to pharynx width 1: 0.4 (1: 0.3-0.4). Oesophagus 465 (410-498) long, extending approximately to level of midbody. Caeca approximately 3 times longer than wide, terminating 465 (419-487) from posterior end representing 34% (31-35%) of BL.

Testis elongate, slightly sigmoid in some, postcaecal, 309 (278-331) long, 80 (74-95) wide, 466 (411-504) from posterior margin of ventral sucker. Posttesticular field representing approximately 6% (4-7 %) of BL. External seminal vesicle saccular to claviform, 104 (97-117) long, 71 (68-81) wide, posterior to ventral sucker. Hermaphroditic sac thick walled, elongate, 233 (186-298) long, 69 (57-88) wide representing 17% (16-19 %) of BL, 2.1 (1.7-2.2) times longer than ventral sucker, with distal portion strongly muscular forming ring with spines; spines 8 in number in distal ring, 7-8 long by 3-6 wide; containing terminal genitalia, internal seminal vesicle 64 (44-79) long, 18 (17-28) wide; prostatic bulb small, 14 (16-29) long, 12 (11-18) wide; male duct short; female duct glandular, 88 (72-99) long; hermaphroditic duct 119 (108-133) long, with male and female ducts uniting at level of distal 1/3 of hermaphroditic sac, strongly muscular, containing thorn-shaped spines. Genital atrium 4 (2-5) deep,

with proximal portion surrounded by muscular ring of hermaphroditic sac. Genital pore medial, at level of anterior margin of ventral sucker.

Ovary subglobular, ventral to level of caecal termination to post caecal, 74 (68-81) long, 70 (63-80) wide, 342 (301-368) from posterior margin of ventral sucker, 54 (33-59) anterior to testis. Laurer's canal not observed. Vitelline masses subglobular, approximately equal in size, contiguous with posterior margin of ovary, contiguous with anterior margin of testis, 46 (44-59) long, 47 (41-57) wide, 404 (374-427) from posterior margin of ventral sucker. Uterus emerging from anterior margin of ovary, winding posteriorly to near posterior margin of body then anteriorly, occupying most of hindbody, mostly dorsal to testis, with proximal portion filled with sperm. Eggs thin-shelled, numerous, with those in distal portion of uterus 31 (29-32) long, 13-15 (13-16) wide; with those in distal portion of uterus with developed miracidia with fused eyespots.

Excretory vesicle I-shaped, elongate saccular, 182 (167-220) long representing 13% (13-15%) of BL; pore terminal.

Type and only known host: *Paramugil georgii* (Ogilby), silver mullet, Mugilidae.

Type-locality: Barred Creek, North of Broome, Western Australia, Australia (17^{o}39'37"S, 122^{o}11'58"E).

Other locality: 6 Mile Creek, Port headland, Western Australia, Australia (20^{o}19'33"S, 118^{o}40'11"E).

Site: Intestine.

Holotype: WAM TBD.

Paratypes: USNM TBD; QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP-561) consensus sequence from 2 specimens from Barred Creek and 2 specimens from 6 Mile Creek.

Remarks. Gen. n. 2 sp. n. 2 can be differentiated from all other species of Gen. n. 2 in processing a cup-shaped, anteriorly directed ventral sucker and a short (>10% of body length) posttesticular field.

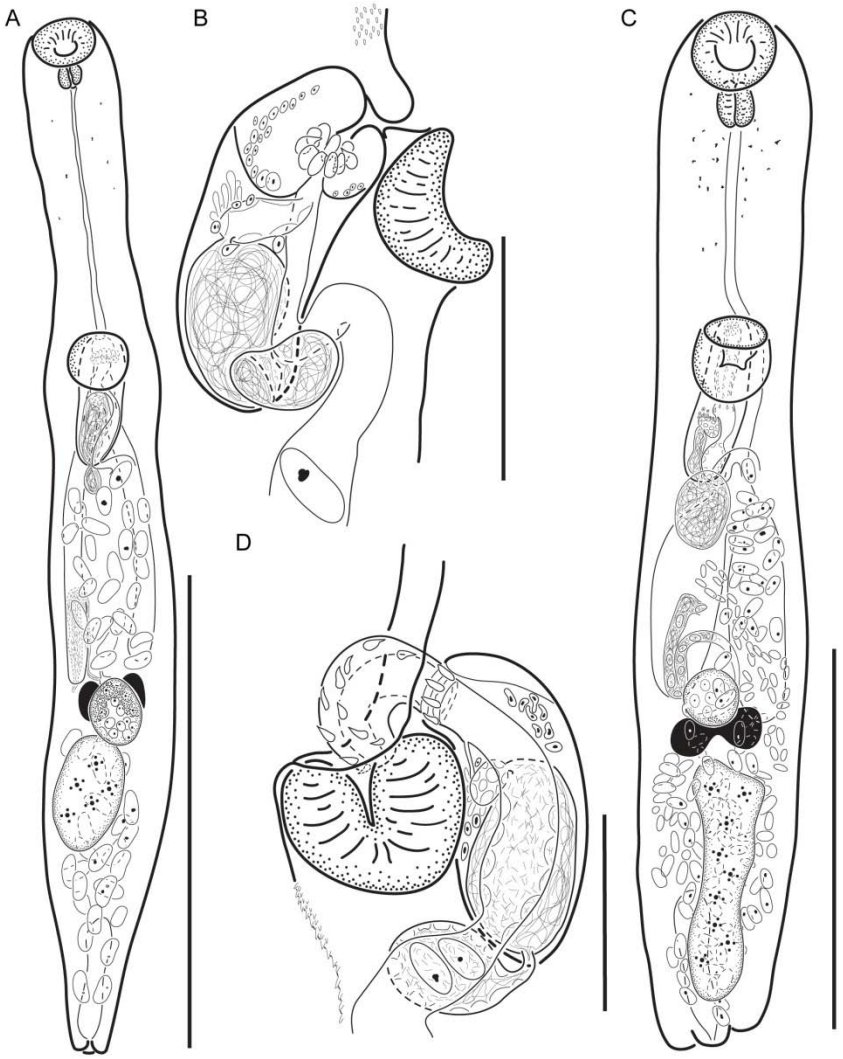


Figure 5.1. A-B. Gen. 2 sp. n. 1. A. Ventral view of holotype. B. Lateral view of hermaphroditic sac. C-D. Gen. 2 sp. n. 2. C. Ventral view of holotype. D. Lateral view of everted hermaphroditic sac. Not all eggs are illustrated for all figures. Scale bars: A, C 500 µm; B, D 100 µm.

Gen. n. 2 sp. n. 3 Figures 5.2A-B.

Description (mesurements based on 1 mature and 2 immature wholemounts): Body elongate, truncated anteriorly, rounded posteriorly, 1,393 (601-1,075) long, 105 (97-105) wide at first third of BL representing 8% (10-16%) of BL. Tegumental spines 1 (1) long, in longitudinal rows with spines becoming fewer to absent at approximately level of midbody. Eyespot pigment apparently lacking. Forebody 188 (157-203) long representing 13% (15-34%) of BL. Hindbody 1,131 (360-847) long representing 81% (60-79%). Oral sucker subglobular, 65 (44-64) long, 69 (46-67) wide. Ventral sucker subglobular, 74 (38-71) long, 80 (36-69) wide. Ratio of oral sucker to ventral sucker widths 1: 1.2 (1: 0.8-1.0). Prepharynx 18 (15-16) long. Pharynx subglobular, 37 (25-42) long, 54 (25-25) wide. Ratio of oral sucker width to pharynx width 1: 0.8 (1: 0.5). Oesophagus 620 (220-480) long, extending approximately to level of midbody. Caeca approximately 18 (4-8) times longer than wide, terminating 249 (191-258) from posterior end representing 18% (24-32%) of BL.

Testis single, elongate, median, intercaecal, 273 (106-2213) long, 73 (64-97) wide, 646 (167-520) from posterior margin of ventral sucker. Posttesticular field representing approximately 16% (10-14 %) of BL. External seminal vesicle elongated, 131 (52-67) long, 32 (21-25) wide, extending posteriorly. Hermaphroditic sac, elongate, posterior to ventral sucker in nearly mature and mature worms, 257 (162-219) long, 62 (51-64) wide representing 18% (20-27 %) of BL, 3.5 (3.1-4.3) times longer than ventral sucker, with distal portion strongly muscular forming ring with spines; spines 10 in number in distal

ring, 7-9 long by 2-3 wide; containing terminal genitalia, internal seminal vesicle 140 (58-97) long, 44 (27-31) wide; prostatic bulb small, 23 (16-18) long, 12 (13) wide; male duct short; female duct glandular, 113 (66-112) long; hermaphroditic duct 66 (55-64) long, with male and female ducts uniting approximately at midlevel of hermaphroditic sac, strongly muscular, containing thorn-shaped spines. Genital atrium, long, 123 (47-147) deep, with proximal portion surrounded by muscular ring of hermaphroditic sac.Genital pore medial, at level of anterior margin of peduncle.

Ovary elongate, approximately at 2/3 of BL, 80 (38-84) long, 52 (40-57) wide, 504 (167-428) from posterior margin of ventral sucker, 63 (0-8) anterior to testis. Laurer's canal not observed. Vitelline masses subglobular, lateral to slightly diagonal, approximately equal in size, contiguous with posterior margin of ovary, contiguous with anterior margin of testis, 56 (23-70) long, 41 (24-40) wide, 605 (131-476) from posterior margin of ventral sucker. Uterus emerging from sinistral to posterior margin of ovary, pretesticular, proximal portion filled with sperm. Eggs thin-shelled, without miracidia with eyespots; with those in distal portion of uterus 26-27 long, 11-12 wide.

Excretory vesicle I-shaped, saccular, 171 (137-143) long representing 12% (13-24%) of BL; pore terminal.

Type and only known host: *Moolgarda perusii* (Valenciennes), longfinned mullet, Mugilidae.

Type-locality: Doyle's Boat Ramp, Fannie Bay, Darwin, Northern Territory, ($12^{o}26'09''S$, $130^{o}49'56''E$).

Site: Intestine.

Holotype: MNT TBD.

Paratypes: USNM TBD; QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP142) consensus sequence from 3 immature specimens.

Remarks. Only the holotype had eggs in the distal portion of the uterus. The larger immature specimen had three developing eggs in the proximal portion of the uterus. All specimens contained sperm in the external seminal vessicle and internal seminal vesicle. This is the first haploporine described with a confined uterus; however, I believe that this species either will attain a larger size or the uterus will eventually fill the hindbody. The largest immature specimen is nearly the same size as the holotype, suggesting that the holotype may not be fully mature. A somewhat restricted uterus was observed in *Pseudodicrogaster* sp. n. 2; although, other specimens that were larger contained a uterus that occupied most of the hindbody (Chapter IV). The lack of eggs containing miracidia with eyespots has also been observed in other haploporines that eventually develop them (Overstreet and Curran 2005, Andres et al. 2014a). Both immature specimens possessed a genital atrium longer than the ventral sucker, but the smaller immature specimen had a genital atrium that was only slightly longer than the ventral sucker and a hermaphroditic sac that was dorsal to the ventral sucker rather than posterior to it. The forebody was approximately equal in length in all specimens, and the hindbody length showed allometric

growth. Gen. n. 2 sp. n. 3 can be differentiated from all other members of Gen. n. 2 in having a genital atrium longer than the ventral sucker, a forebody that is <10% of the body length, and an excretory vesicle that is elongated.

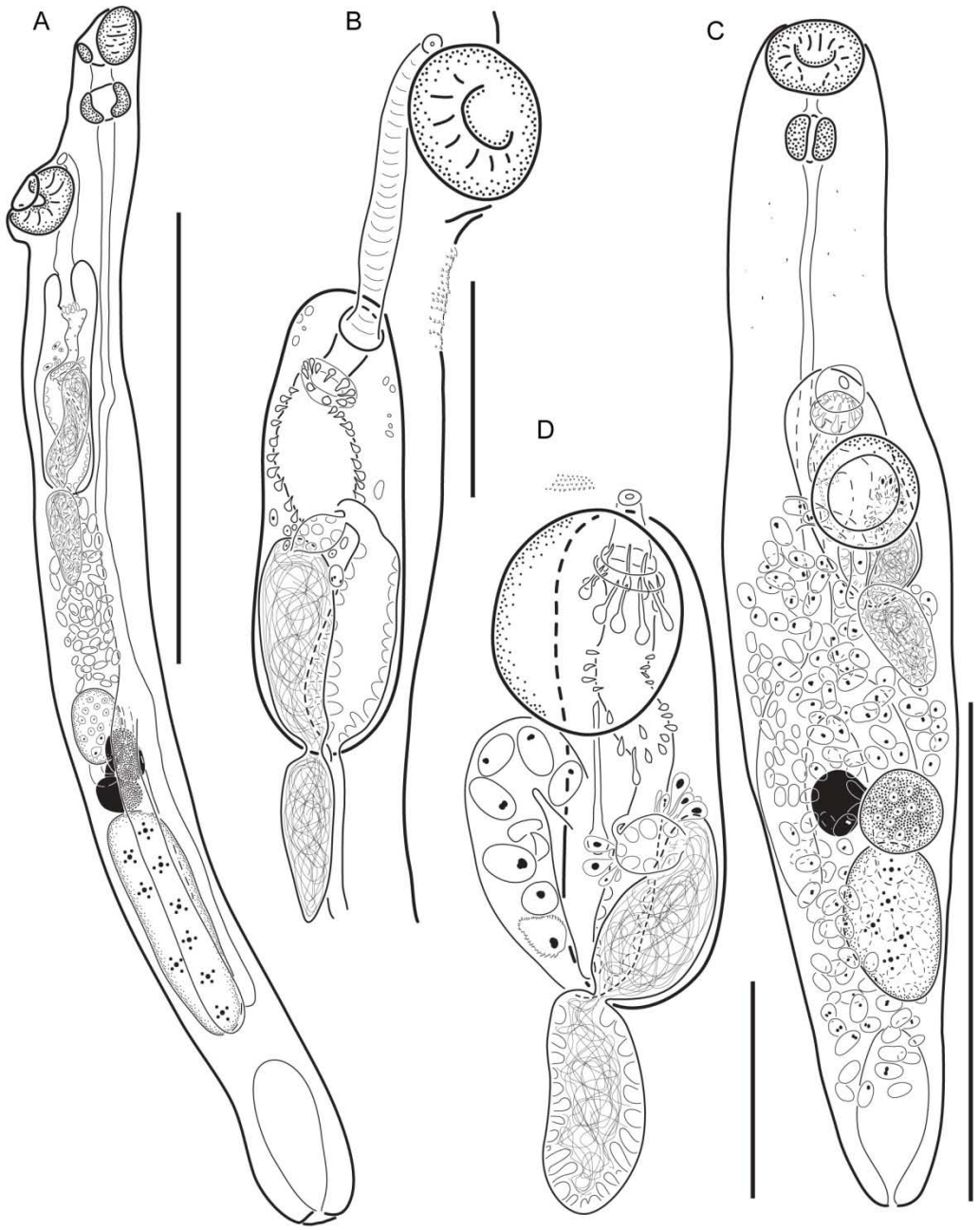


Figure 5.2. A-B. Gen. 2 sp. n. 3. A. Lateral view of holotype. B. Lateral view of hermaphroditic sac, showing highly muscular distal portion of the hermaphroditic sac and long genital atrium. C-D. Gen. 2 sp. n. 4 Not all eggs are illustrated. C. Ventral view of holotype. D. Ventral view of hermaphroditic sac. Scale bars: A, C 500 µm; B, D 100 µm.

Gen. n. 2 sp. n. 4 Figures 5.2C-D.

Description (mesurements based on 7 wholemounts): Body elongate, fusiform, 1,183 (1,054-1,345) long, 218 (198-224) wide at first third of BL representing 18% (17-19%) of BL. Tegumental spines 1-2 (1-2) long, in longitudinal rows. Eyespot pigment apparently absent. Forebody 416 (386-434) long representing 35% (32-37%) of BL. Hindbody 655 (558-787) long representing 55% (53-59%). Oral sucker 73 (68-76) long, 98 (82-105) wide. Ventral sucker subglobular, 112 (110-124) long, 114 (107-120) wide. Ratio of oral sucker to ventral sucker widths 1: 1.2 (1: 1.1-1.3). Prepharynx 23 (14-34) long. Pharynx, subglobular 46 (38-49) long, 52 (47-67) wide. Ratio of oral sucker width to pharynx width 1: 0.5 (1: 0.4-0.7). Oesophagus 509 (478-571) long, extending approximately to level of 2/5 to midbody. Caeca approximately 5 times longer than wide, terminating 318 (298-354) from posterior end representing 27% (24-28%) of BL.

Testis intercaecal, 160 (145-168) long, 99 (80-108) wide, 288 (245-297) from posterior margin of ventral sucker. Posttesticular field representing approximately 18% (17-21%) of BL. External seminal vesicle saccular, glandular, 110 (101-124) long, 59 (52-63) wide, posterior to ventral sucker. Hermaphroditic sac, elongate, 247 (188-291) long, 103 (97-125) wide representing 21% (18-22 %) of BL, 2.2 (1.7-2.1) times longer than ventral sucker, with distal portion strongly muscular forming ring with spines; spines 10 in number in distal ring, approximately 28 (27-31) long by 6 (4-7) wide; containing terminal genitalia, internal seminal vesicle 77 (71-98) long, 40 (33-47) wide; prostatic bulb small, 26

(19-41) long, 25 (21-35); male duct short; female duct glandular, 94 (88-101) long; hermaphroditic duct 119 (97-134) long, with male and female ducts uniting approximately at midlevel of hermaphroditic sac, strongly muscular, containing tiny spines, containing single large spine; large spine falcate, 58 (51-68) long, 16 (14-17) wide at base. Genital atrium 13 (11-18) deep, with proximal portion surrounded by muscular ring of hermaphroditic sac. Genital pore medial, 16 from anterior margin of ventral sucker.

Ovary medial to sinistral, postcaecal, 78 (74-85) long, 75 (71-80) wide, 217 (171-212) from posterior margin of ventral sucker, contiguous with anterior margin of testis, intercaecal. Laurer's canal not observed. Vitelline masses subglobular, approximately equal in size, contiguous with ovary, contiguous with anterior margin of testis, 67 (64-78) long, 55 (42-61) wide, 218 (266-287) from posterior margin of ventral sucker. Uterus extensive, occupying most of hindbody anterior to near posterior margin of ventral sucker, proximal portion filled with sperm. Eggs thin-shelled, numerous; with those in distal portion of uterus 30-32 (29-34) long, 16-17 (16-18) wide; with those in distal portion of uterus with developed miracidia with fused eyespots.

Excretory vesicle I-shaped, saccular, 175 (134-197) long representing 15% (13-15%) of BL; pore terminal.

Type and only known host: *Moolgarda seheli* (Forsskål), bluespot mullet, Mugilidae.

Type-locality: Eli Creek, Hervey Bay, Queensland, Australia (25^{o}15'45"S, 152^{o}48'27"E).

Site: Intestine.

Holotype: QM TBD.

Paratypes: USNM TBD; QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP-154) from a single specimen.

Remarks. This species can be differentiated from all other species of Gen. n. 2 in possessing a hermaphroditic sac that contains a single, large (>45 µm) spine.

Gen. n. 2 indicus (Rekharani et Madhavi, 1985)

syn. *Haploporus indicuse* Rekharani et Madhavi, 1985.

Type-host: *Moolgarda cunnesius* (Valenciennes), longarm mullet, Mugilidae (as *Valamugil cunnesius* [Valenciennes]).

Type-locality: locations around Visakhapatnam, Andhra Pradesh, India, or Chilka Lake, India.

Site: Intestine.

Holotype: BMNH 1984.6.28.20.

Remarks. Reckharani and Madhavi (1985) described and illustrated Gen. n. 2 *indicus* without spines lining the hermaphroditic duct or ‘genital atrium’. In the same publication they described *H*. *pseudindicus*, also without spines; although, Blasco-Costa et al. (2009b) examined the holotype of *H*. *pseudindicus* and confirmed that spines were present. Reckharani and Madhavi's (1985) illustration (Figure 2 of Reckharani and Madhavi [1985]) shows small stipples lining the hermaphroditic duct, and they may represent spines. Regardless, if spines are

present or not in the hermaphroditic duct, the overall morphology and geography of Gen. n. 2 *indicus* fit within my generic diagnosis of Gen. n. 2 and can be differentiated from all other members of the genus in having the intestinal bifurcation at or anterior to the level of the anterior margin of the ventral sucker.

Gen. n. 2 magnisaccus (Machida, 1996) n. comb.

syn. *Haploporus magnisaccus* Machida, 1996.

Type-host: *Moolgarda seheli*, bluespot mullet, Mugilidae (originally reported as *Crenimugil crenilabis* [Forsskål] and corrected by Machida [2003]).

*Other host*s: *Mugil cephalus*(?) Linnaeus, flathead grey mullet; cf. *Chelon subviridis* (Valenciennes), greenback mullet, both Mugilidae.

Type-locality: off Nago, Okinawa Prefecture, Japan.

Other locality: Ambon, Indonesia; Nha Trang, Khánh Hòa Province, Vietnam.

Site: Intestine.

Holotype: NSMT-Pl 4290.

Paratypes: NSMT-Pl 4290; NSMT-Pl 4317.

Material examined: 8 specimens from (?) *Chelon subviridis*.

Supplemental material: Tegumental spines covering periphery of ventral sucker. Caeca approximately 2.5-4 times longer than wide. Testis elongate, elliptical to irregular. Genital atrium 25-45. Genital pore 5-11 from anterior margin of ventral sucker. USNM TBD; BMNH TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-767) from a single specimen.

Remarks. The Vietnamese specimens agree with the original description of Gen. n. 2 *magnisaccus* by Machida (1996) and the additional data provided by Blasco-Costa et al. (2009b). In particular, the arrangement of spines surrounding the muscular, distal portion of the hermaphroditic sac (termed a genital atrium by Blasco-Costa et al. [2009b], a definition with which I do not agree) and the spines lining the everted, hermaphroditic duct. Machida (1996) originally described the genital pore as "some distance anterior to acetabulum" (128); however, in the specimens from Vietnam the genital pore is 5-11 anterior from the anterior margin of the ventral sucker. I consider this difference to be minor, particularly as the specimens of Machida (1996) were fixed under some pressure. Gen. n. 2 *magnisaccus* is differentiated from the other species of Gen. n. 2 by possessing an elongated external seminal vesicle and a hermaphroditic sac with a combination of a well-developed prostatic complex and a hermaphroditic duct lined with small spines.

Gen. n. 2 megasacculum (Liu, Wang, Peng, Yu, et Yang, 2004) n. comb.

syns. *Saccocoelium megasacculum* Liu, Wang, Peng, Yu, et Yang, 2004; *Eliptobursa megasacculum* (Liu, Wang, Peng, Yu, et Yang, 2004) Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009.

Type-host: *Liza affinis* (Günther), eastern keelback mullet, Mugilidae (as *Liza carinatus* [Cuvier et Valenciennes]).

Other host: (?) *Chelon subviridis* (Valenciennes), greenback mullet, Mugilidae.

Type-locality: off Xiamen, Fujian Province, China.

Other locality: Nha Trang, Khánh Hòa Province, Vietnam.

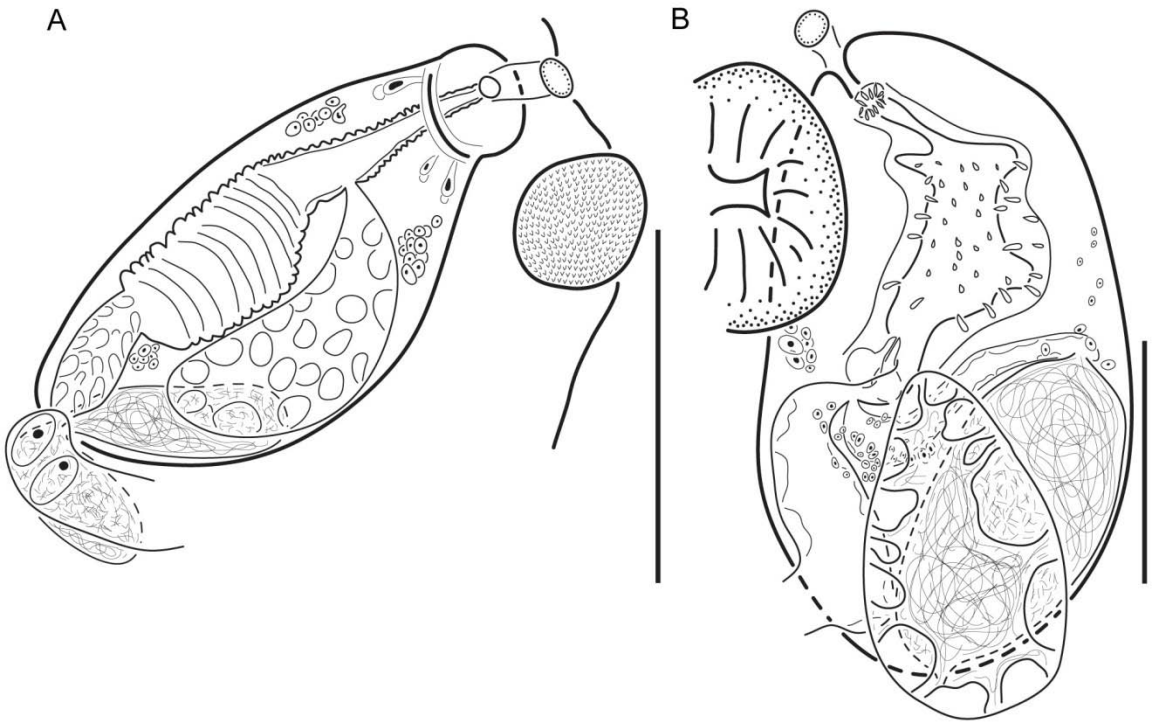
Site: Intestine.

Holotype: FJXM 20010210-1.

Paratypes: FJXM 200200724-2-10, 20010210-3, 20010210-3; USNPC 93681.

Material examined: USNPC 93681; 18 specimens from (?) *Chelon subviridis*.

Supplemental material: Figures 5.3A, 5.4. Body 523-616. Tegumental spines on ventral surface of forebody lacking from level of pharynx to anterior margin of ventral sucker; covering ventral sucker. Genital pore 16-27 from anterior margin of ventral sucker. USNM TBD.



Figures 5.3. A. Lateral view of hermaphroditic sac of Gen. n. *megasacculum* and tegumental spines on the ventral sucker. B. Lateral view of the hermaphroditic sac of Gen. n. 3 mugilis. Not all eggs are on both figures. Scale bars: 9, 10 100 µm.

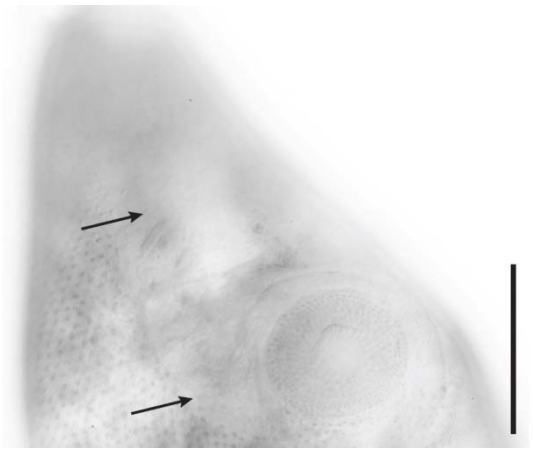


Figure 5.4. Micropictograph of Gen. n. 2 *megasacculum* showing the ventral surface of the forebody lacking spines (arrows) and spination of the ventral sucker. Scale bar 100 µm.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-767) from a single specimen.

Remarks. Liu et al. (2010) provided a host-parasite list for digeneans of Chinese marine fishes and listed the host for *E. megasacculum* as *Liza haematocheila* (Temminck et Schlegal) (as *Chelon haematocheilus* [Temminck et Schlegal]), citing the original description as the source. The Vietnamese specimens morphologically agree with the description by Liu et al. (2004). All specimens examined (including the paratype) have a ‘naked chest’ (lacking spines on the ventral surface of the forebody between the level of the pharynx to the anterior margin of the ventral sucker) and a ventral sucker covered in tegument spines, both characteristics were not described in the original description. Blasco-Costa et al. (2009e) transferred *S. megasacculum* to *Elliptobursa*, apparently agreeing with Madhavi (2008) that *Elliptobursa* belongs in the Haploporidae. I do not agree with either of these decisions. Wu et al. (1996) described and illustrated the distal portion of the uterus of the type

species, *Elliptobursa singlorchis* Wu, Lu, et Zhu, 1996, as separate from the cirrus sac. The hermaphroditic sac of Gen. n. 2 *megasacculum* lacks spines, but otherwise the species agrees with the generic diagnosis. Gen. n. 2 *megasacculum* can be differentiated from the other members of the genus based on the ventral surface of the forebody lacking spines, the hermaphroditic duct lacking spines, and the ventral sucker covered in spines.

Gen. n. 2 mugilis (Liu et Yang, 2002) n. comb.

syn. *Haploporus mugilis* Liu et Yang, 2002.

Type-host: *Moolgarda engeli* (Bleeker), kanda, Mugilidae (as *Valamugil engeli* [Bleeker]).

Other host: (?) *Chelon subviridis* (Valenciennes), greenback mullet, Mugilidae.

Type-locality: Xiamen, Fujian Province, China.

Other locality: Daya Bay, Guangdong Province, China (22°43'N, 114°32'E).

Site: Intestine.

Holotype: FJXM 20010210-1.

Paratypes: FJXM 20010210-2-4; BMNH 2001.8.6.1-2; USNPC 91707; Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic D-456.

Material examined: USNPC 91707; 1 specimen from Daya Bay, China.

Supplemental material: Figure 5.3B. External seminal receptacle glandular. USNM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP-610) consensus sequence from 3 identical specimens.

Remarks. The single specimen collected by Eric Pulis has the tegument ruptured at the level of the caecal bifurcation and an artificial constriction near the posterior margin of the body. However, the specimen is in good enough condition to compare measurements with those of the paratype examined, and is within the range provided in the description by Liu and Yang (2002). The only departure from the original description of Gen. n. 2 *mugilis* is that the ovary is contiguous with the anterior margin of the testis. I attribute this difference to the artificial constriction of the hindbody and an anterior displacement of the testis. The most striking morphological feature of this species is the paired, broad, round based, falcate spines in the posterior portion of the hermaphroditic duct. The spine pair is dorsal to the level of approximately where the male and female ducts (termed a metraterm by Liu and Yang [2002], a definition with which I disagree) join (Figure 5.3B).

Gen. n. 2 spinosus (Machinda, 1996)

syn. *Haploporus spinosus* Machida, 1996.

Type and only known host: *Moolgarda seheli*, bluespot mullet, Mugilidae (originally reported as *Crenimugil crenilabis* [Forsskål] and corrected by Machida [2003]).

Type-locality: off Nago, Okinawa Prefecture, Japan.

Other locality: Ambon, Indonesia; Vietnam.

Site: Intestine.

Holotype: NSMT-Pl 4162.

Paratypes: NSMT-Pl 4162; NSMT-Pl 4365; NSMT-Pl 4709.

Remarks. Machida (1996) differentiated this species from the other species of *Haploporus* in possessing two groupings of spines along the hermaphroditic duct; one group of spines in a ring around the distal portion of the hermaphroditic duct and the other group of spines in two pairs located approximately at the level of the hermaphroditic duct. Blasco-Costa et al. (2009b) examined paratypes of Gen. n. 2 *spinosus* and provided a detailed illustration of the hermaphroditic sac and provided additional measurements of the spines contained within the hermaphroditic sac. Most members I consider to belong in Gen. n. 2 contain an armed hermaphroditic duct; however, no other species has this particular arrangement of spines. Gen. n. 2 sp. n. 4 possesses a single, long spine in its hermaphroditic sac and is the only species to possess a spine close to the length of those in Gen. n. 2 *spinosus*.

Molecular analysis

No intraspecific variation in the sequences of the ITS and partial 28S rDNA was observed in any of the species with replicates. Lengths of the ITS and partial 28S rDNA sequences are found in Table 5.1. Pairwise comparison of the 18S and ITS1 sequence data of the seven species of Gen. n. 2 are shown in Table 5.2. Pairwise comparison of the ITS2 and partial 28S sequence data of the seven species of Gen. n. 2 are found in Table 5.3. The 5.8S sequences were

identical for five of the species of Gen. n. 2, with that of Gen. n. 2 *megasacculum* being different from the rest by a single bp (0.6%).

Table 5.1

Base pair lengths of sequences for the partial 18S rDNA, ITS1, 5.8S rDNA, ITS2, and partial 28S of seven species of Gen. n. 2.

	18S	ITS1	5.8S	ITS2	28S
Gen. n. 2 sp. 1	108	549	158	295	1,390
Gen. n. 2 sp. 2	108	550	158	283	1,389
Gen. n. 2 sp. 3	108	553	158	282	1,389
Gen. n. 2 sp. 4	108	553	158	283	1,387
Gen. n. 2 *magnisaccus*	108	547	158	276	1,389
Gen. n. 2 *megasacculum*	108	578	158	282	1,386
Gen. n. 2 *mugilis*	108	548	158	283	1,391

Table 5.2

Pairwise comparisons of percent nucleotide similarity and number of base pair differences (in parentheses) of the partial 3' end of the 18S (below the diagonal) sequences and ITS1(above the diagonal) sequences of the species of Gen. n. 2.

	Gen. n. 2 sp. 1	Gen. n. 2 sp. 2	Gen. n. 2 sp. 3	Gen. n. 2 sp. 4	Gen. n. 2 *magnisaccus*	Gen. n. 2 *megasacculum*	Gen. n. 2 *mugilis*
Gen. n. 2 sp. 1	-	86.1 (66)	85.9 (77)	87.7 (67)	83.6 (86)	87.1 (70)	84.6 (84)
Gen. n. 2 sp. 2	96.3 (4)	-	90.1 (50)	89.9 (55)	88.0 (63)	89.9 (55)	91.2 (48)
Gen. n. 2 sp. 3	99.1 (1)	97.2 (3)	-	90.3 (53)	87.1 (68)	89.2 (55)	94.5 (30)
Gen. n. 2 sp. 4	96.3 (4)	100	97.2 (3)	-	87.4 (66)	90.7 (51)	89.6 (57)
Gen. n. 2 *magnisaccus*	97.2 (3)	95.4 (5)	98.1 (2)	95.4 (5)	-	87.2 (67)	87.5 (65)
Gen. n. 2 *megasacculum*	96.3 (4)	100	97.2 (3)	100	95.4 (5)	-	88.8 (61)
Gen. n. 2 *mugilis*	99.1 (1)	97.2 (3)	100	97.2 (3)	98.1 (2)	97.2 (3)	-

Table 5.3

Pairwise comparisons (excluding gaps) of percent nucleotide similarity and number of base pair differences (in parentheses) of the ITS-2 (below the diagonal) and partial 28S (above the diagonal) sequences of the species of Gen. n. 2.

	Gen. n. 2 sp. 1	Gen. n. 2 sp. 2	Gen. n. 2 sp. 3	Gen. n. 2 sp. 4	Gen. n. 2 *magnisaccus*	Gen. n. 2 *megasacculum*	Gen. n. 2 *mugilis*
Gen. n. 2 sp. 1	-	97.4 (36)	95.7 (60)	96.0 (56)	95.1 (65)	95.2 (62)	95.7 (60)
Gen. n. 2 sp. 2	87.6 (35)	-	95.7 (60)	96.8 (45)	96.0 (55)	96.0 (55)	95.9 (57)
Gen. n. 2 sp. 3	85.8 (40)	91.8 (23)	-	95.3 (65)	95.9 (57)	94.9 (70)	98.6 (20)
Gen. n. 2 sp. 4	85.9 (40)	90.2 (24)	87.9 (34)	-	94.8 (72)	95.9 (57)	95.3 (65)
Gen. n. 2 *magnisaccus*	82.3 (49)	86.9 (37)	90.0 (28)	86.5 (38)	-	94.7 (73)	95.8 (58)
Gen. n. 2 *megasacculum*	87.3 (43)	92.0 (22)	89.8 (28)	92.0 (22)	85.5 (40)	-	95.2 (66)
Gen. n. 2 *mugilis*	86.5 (38)	91.5 (24)	95.7 (12)	88.3 (33)	88.6 (32)	88.7 (31)	-

The alignment of partial 28S rDNA sequences of the seven species of Gen. n. 2 and related species from GenBank was 1,127 characters long with 616 conserved sites, 511 variable sites, and 409 informative sites. The BI analysis (Figure 5.5) of those sequences incorporated *A. sigani* as the outgroup, *P. ishigaki,* and an ingroup of 46 haploporids. *Hapladena nasonis* was resolved as the basal taxa, with *Cadenatella* as the poorly supported sister group to a large clade containing the rest of the haploporids. The large clade contained two main subclades; 1) of the Haploporinae and 2) made up of *Intomugil* spp. + *Saccocoelioides* sp. and members of Forticulcitinae Blasco-Costa, Balbuena,

Kostadinova, et Olson, 2009, + members of Waretrematinae Srivastava, 1937. The seven species of Gen. n. 2 were resolved as a polytomy sister to the other 13 haploporine species.

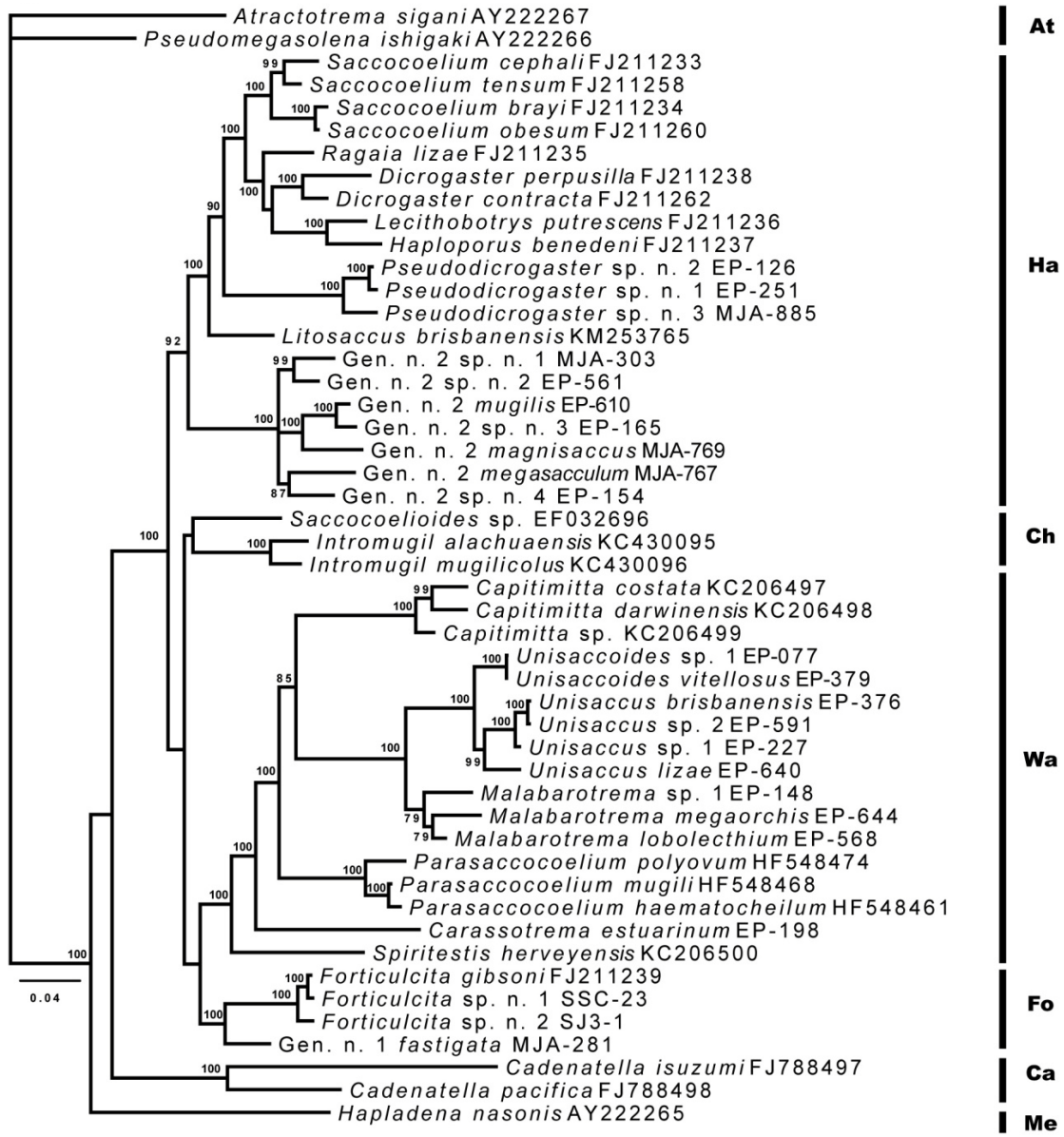


Figure 5.5. Phylogenetic relationships among members of the Haploporidae resulting from Bayesian inference analysis of partial sequences of 28S rDNA gene (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100) revealing Gen. n. 2 as the sister group to the rest of the Haploporinae. Support values of <75 not shown. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellinae; Ch = Chalcinotrematinae; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasoleninae; Wa = Waretrematinae.

Discussion

Members of Gen. n. 2

Blasco-Costa et al. (2009b) recognized that the Indo-Pacific species of *Haploporus* from mugilid hosts that they referred to as ‘‘the species complex from *Valamugil* spp.”, did not belong in *Haploporus* and that those species share several morphological features. However, Blasco-Costa et al. (2009b) stated that the members of “the species complex from *Valamugil* spp.” (118) could not be attributed to another haploporine genus, and thus left them as *incertae sedis* with respect to their generic affiliation. Based on material I and others have collected from Australia, China, and Vietnam, I erect Gen. n. 2 for four newly described species, four species that Blasco-Costa et al. (2009a) considered *incerte sedis*, and Gen. n. 2 *megasacculum* that Blasco-Costa et al. (2009e) attributed to *Elliptobursa*.

Wu et al. (1996) erected *Elliptobursa* for *E. singlorchis* from *Liza affinis* (Günther) (as *Mugil affinis* Günther) off Huangfu Town, Huidong County, Guangdong Province, China, and originally attributed the genus to the Monorchiidae. Wu et al. (1999) described a second species of *Elliptobursa*, *Elliptobursa attenuatus* Wu, Lü, et Chen, 1999, from *Mugil parvus* (Oshima) also off Huangfu town, but the exact host species is unclear. *Liza parva* Oshima is a junior synonym of *Chelon macrolepis* (Froese and Pauly 2014), thus the host may be *C*. *macrolepis*; however, Liu et al. (2010) listed *Mugil cephalus* as the host for *E*. *attenuatus*. Furthermore, Lü (1993) described *Saccocoelioides huidongensis* Lü, 1993 from *M*. *parvus* and later Liu et al. (2010) stated that it

was from *Mugilogobius parvus* (Oshima). Both species of *Elliptobursa* are described and illustrated as having a uterus that empties into a genital atrium separate from the male terminal genitalia (called a cirrus sac by Wu et al. 1996, 1999). Madhavi (2008) transferred both *Elliptobursa* and *Allomonorchis* to the Haploporidae "as evidenced by the presence of a single testis, a long external seminal vesicle, a well-developed prostatic complex, and a long hermaphroditic duct wrongly interpreted as a cirrus" (146). A well-developed prostatic complex and a single testis are seen in at least 17 monorchhid genera. An external seminal vesicle is not generally considered a character of the monorchiids, but it is seen in members of *Allolasiotocus* Yamaguti, 1959. I am hesitant to interpret the cirrus sac in any other way than it is illustrated and described. Additionally, *E. singlorchis* is described as having a saccular excretory vesicle with arms extending to the level of the posterior margin of the ventral sucker. Therefore, I consider *Elliptobursa* and *Allomonorchis* as *genera inquirendae*, transfer *E*. *megasacculum* to Gen. n. 2, and consider the rest of the members of *Elliptobursa* and *Allomonorchis* as *species inquirendae*.

Blasco-Costa et al. (2009b) considered *H*. *musculosaccus*, *H*. *pacificus*, *H*. *pseudoindicus*, *Lecithobotrys suezcanali* Nisreen Ezz El-Dien, Abdel-Rahman, El-Gawady, Imam, et Fahmy, 1990, and *Lecithobotrys vitellosus* Gharma et Gupta, 1970 as *species inquirendae*. Of those, only *H*. *pseudindicus* sp. inq. seems likely as a candidate for placement within Gen. n. 2. Blasco-Costa et al. (2009b) examined the holotype of *H*. *pseudoindicus* and provided a more detailed illustration of the hermaphroditic sac. They considered the species close

to the species from *Valamugil* spp. complex; however, they stated "the state of the Indian specimen does not permit a decision to be made" (Blasco-Costa et al. 2009b: 116). I did not examine the holotype nor collect any Australian specimens close to *H. pseudoindicus*; thus, I agree with Blasco-Costa et al. (2009b) in considering *H. pseudindicus species inquirende*. Additionally, I agree with Blasco-Costa et al. (2009b) in considering *L*. *vitellosus* and *L*. *suezcanali* as *species inquirendae*. Both species are poorly described and illustrated, and each is also described as having a Y-shaped excretory vesicle and a prepharynx nearly as long as the oesophagus. I consider neither to be characters of Gen. n. 2.

I also agree with Blasco-Costa et al. (2009b) in considering *H. pacificus* and *H. musculosaccus* as *species inquirende*. *Haploporus pacificus* departs from both *Haploporus* and Gen. n. 2. in possessing vitellarium as two masses of four to five short, compact to elongate, branch-like lobes rather than two symmetrical, globular to subglobular masses; a prepharynx nearly as long to longer than the pharynx rather than shorter than the pharynx; and a sctophagid rather than mugilid host. *Haploporus pacificus* further departs from Gen. n. 2 in possessing a Y-shaped excretory vesicle that extends to the level of the midbody rather than an I-shaped excretory vesicle that extends to the level of the last third of the body length. Thus, even though the generic name *Neohaploporus* is available, I refrain from using that name for the species treated herein because molecular data of *H. pacificus* will likely reveal that species to be distinct from both *Haploporus* and Gen. n. 2.

Machida (2003) described *H*. *musculosaccus* from *Moolgarda seheli* off Nago, Okinawa Prefecture, Japan. His description and illustration of *H*. *musculosaccus* sp. inq. lead Blasco-Costa et al. (2009b) to suggest that *H*. *musculosaccus* does not belong in a natural group with the other Indo-Pacific *Haploporus* species; I agree with that decision. *Haploporus musculosaccus* sp. inq. possesses morphological features that I consider outside of Gen. n. 2, including caeca that terminates blindly at approximately the first quarter of body length, an esophagus that is short, the distal portion of the uterus extending into the forebody, a hermaphroditic sac that consists of two portions, and an infundibuliform oral sucker that is ringed by sensory papillae. Machida's (2003) description of the hermaphroditic sac in “two portions” (126) is misleading, especially when comparing his illustrations of the hermaphroditic sac (Figure 2 vs. Figure 4 of Machida [2003]). His lateral illustration (Figure 4 of Machida [2003]) of the hermaphroditic sac is very similar to the hermaphroditic sac of *Litosaccus brisbanensis* (Martin, 1974) illustrated by Andres et al. (2014; Figure 3). *Haploporus musculosaccus* sp. inq. also shares a terminal, infundibuliform oral sucker with small papillae surrounding periphery and a relatively simple (i.e., without large spines or an enlarged prostatic complex) hermaphroditic sac. However, *H*. *musculossacus sp. inq.* is illustrated as having two entire, subglobular, compact masses of vitellaria, not the 'grape-like' vitellarium processed by *L. brisbanensis*. Machida's (2003) specimens were fixed under cover slip pressure that may have shifted the terminal genetalia more anterior (as illustrated in Figure 2 of Machida [2003]). I believe that once additional material

of *H. musculossacus* is collected (preferably coupled with molecular data), this species will likely be best accommodated in *Litosaccus* Andres, Pulis, Cribb, et Overstreet, 2014.

Molecular data

The position of *H. nasonis* outside of the poorly supported *Cadenatella* + 'mugilid' haploporids was consistent with previous analyses (Andres et al. 2014a, Chapter III, IV). This phylogenetic hypothesis deviates from those previous analyses, in the 'mugilid' taxa were resolved in two subclades. The Haploporinae was resolved as sister to a poorly supported clade of *Intromugil* spp. + *Saccocoelioides* sp and Forticulcitinae + Waretrematinae. This is also the first phylogenetic hypothesis where the *Intromugil* spp. + *Sacccoelioides* sp. clade was not the sister taxa to the haploporines. Pulis (2014) advocated the move of *Intromugil* to the Chalcinotrematinae based on BI analysis of partial 28S rDNA sequences, which I tentatively agree with as well; however, the molecular representation of the Chalcinotrematinae is depauperate.

My BI analysis (Figure 5.5) revealed Gen. n. 2 as the sister group to the rest of the haploporines but the hypothesis failed to resolve the intraspecific relationships of the species of Gen. n. 2. The polytomy consisted of Gen. n. 2 sp. n. 1 + Gen. n. 2 sp. n. 2; Gen. n. 2 *mugilis* + Gen. n. 2 sp. n. 3 and Gen. n. 2 *magnisaccus*; and Gen. n. 2 *megasacculum* + Gen. n. 2 sp. n. 4. The morphological variation observed for members within Gen. n. 2 (e.g., body shape, complexity of the hermaphroditic sac, tegumental spination, seminal receptacle) and the lack of phylogenetic resolution may indicate that Gen. n. 2

may eventually be found to be paraphyletic. However, at this time there is not a clear morphological character set, geographic pattern, nor final host association that would justify separating the seven treated species into three separate genera. In particular, the close association of Gen. n. 2 *megasacculum*, which lacks an armed hermaphroditic duct, with Gen. n. 2 sp. n. 4, which has an armed hermaphroditic duct and a large spine in the hermaphroditic sac, is surprising. The lack of spines along the hermaphroditic duct of Gen. n. 2 *megasacculum* may be the result of secondary loss of spines; however, additional species of Gen. n. 2 in a phylogenetic framework are needed to assess this.

Blasco-Costa et al. (2009b) originally suggested that the members of Gen. n. 2 were likely closely related, referring to them as the 'species from *Valamugil* spp.', thus perhaps a final host association may eventually be determined. However, the systematics of the Mugilidae (Cuvier) has undergone a major revision. Durand et al. (2012a, b) used phylogenetic analyses of sequences from three mtDNA loci of mugilids to demonstrate that *Chelon*, *Moolgarda*, and *Valamugil* are paraphyletic, and *Liza* Jordan et Swain is polyphyletic. The phylogenetic hypothesis by Durand et al. (2012b) demonstrated that *M*. *seheli* (type host for Gen. n. 2 sp. n. 4) was paraphyletic, and in a clade with *V*. *buchanani* (type host for Gen. n. 2 sp. n. 1) and *Crenimugil crenilabis* (type host for Gen. n. 2 *magnisaccus* and Gen. n. 2 *spinousus*). Their analysis recovered *M. perusii* (type host for Gen. n. 2 sp. n. 3) in a clade with *M. cunnesius* (type host for Gen. n. 2 *indicus*) and *M*. *engeli* (type host for Gen. n. 2 n. *mugilis*). *Chelon subviridis* (the host identification tentatively applied to the hosts from which my

Vietnamese and Chinese specimens were obtained from) was in a clade with *C. macrolepis* (type host for *H*. *pseudoindicus* sp. inq.), *L. affinis*, and *L. haematocheila* (both of which are potentially the type host for Gen. n. 2 *megasacculum*). Thus, no host trend can yet be applied to any of the species of Gen. n. 2. Additionally, the host data that I provide for Gen. n. 2 *magnisaccus*, Gen. n. 2 *megasacculum*, and Gen. n. 2 *mugilis* is questionable. I have little confidence that *C. subviridis* is the correct host identification for those three species of Gen. n. 2; no ichthyologist was available at the time of collection to ensure proper identification of the host species and three different species of mullet keyed out to *C. subviridis* (Overstreet per. comm.; Pulis pers comm.). Therefore, additional species of Gen. n. 2 that are coupled with molecular data, are needed to help clarify the intraspecific relationships of Gen. n. 2. and test whither a potential co-evolutionary pattern can be discerned. Particularly, as this clade does not seem to be represented in members of the *Mugil cephalus* complex, with only Gen. n. 2 *magnisaccus* being tentatively reported from *M*. *cephalus* (Machida 1996).

Haploporid species and generic concepts have consistently been shown to contain more diversity when coupled with molecular data than based on morphology alone (Blasco-Costa et al. 2010, Pulis and Overstreet 2013, Chapter IV), and occasionally two or more sympatric species have been found to occur in the same host species (Blasco-Costa et al. 2010, Chapter III). Because of this underestimation of species diversity and the problems associated with mugilid systematics, I highly encourage all future haploporid workers to heat kill their

specimens with near boiling (but not boiling) water or physiological saline solution, and then preserve all specimens in 70% molecular grade ethanol (Justine et al. 2012). For this and previous studies (Pulis 2014, Chapter III, Chapter IV), worms intended for sequencing analysis were placed in room temperature or cold ethanol. This causes the specimens to be almost worthless for morphological examination and increases the difficulty in matching the correct specimen for sequencing with the correct morphological voucher when multiple species of haploporids occur within the same host. When all specimens are fixed in this way, individual speciemens can be selected for use as a hologenophore (*sensu* Pleijel et al. [2008]) and can be used for scanning electron microscopy.

Despite the improved molecular representation the family has recently received (e.g., Blasco-Costa et al. 2009a, Pulis and Overstreet 2013, Besprozvannykh et al. 2014, Bray et al. 2014), the Chalcinotrematinae, Megasoleninae, and Waretrematinae lack a molecular representative of the type genus, let alone the type species. Therefore, the family still requires considerable revision, particularly as it applies to the deeper portions of the haploporiod tree.

Key to Gen. n. 2

1a. Ventral surface of forebody between level of pharynx and ventral sucker with no spines or a few irregularly spaced spines; hermaphroditic duct without spines..... Gen. n. 2 *megasacculum* (Liu, Wang, Peng, Yu, et Yang, 2004)

b. Ventral surface of forebody covered in spines in longitudinal rows 2

2a. Ventral sucker cup-shaped; ventral surface oriented towards anterior end of body.....Gen. n. 2 sp. n. 2

b. Ventral sucker not as described above; ventral surface of ventral sucker facing ventrally..... 3

3a. Hermaphroditic sac containing a single large (> 50 µm) spine, with distal end extending into hermaphroditic duct; hermaphroditic duct lined with rows of smaller (< 30 µm) spines ..... Gen. n. 2 sp. n. 4

b. Hermaphroditic sac not containing a single large spine but may contain 2 to 4 spines 4

4a. Hermaphroditic sac containing four enlarged spines (> 60 µm) at level of approximately mid-hermaphroditic duct..... ..... Gen. n. 2 *spinosus* (Machinda, 1996)

b. Hermaphroditic duct without four enlarged spines..... 5

5a. Hermaphroditic duct with paired, falcate spines in the proximal portion of the hermaphroditic duct; dorsal to level of approximately where male and female ducts unite..... Gen. n. 2 *mugilis* (Liu et Yang, 2002)

b. Hermaphroditic duct either lined with spines or not but lacking the paired spines described above 6

6a. Intestinal bifurcation anterior to or at level of anterior margin of ventral sucker ..... Gen. n. 2 *indicus* (Rekharani et Madhavi, 1985)

b. Intestinal bifurcation posterior to or at posterior margin of ventral sucker..... 7

7a. External seminal vesicle saccular, short..... Gen. n. 2 sp. n. 1

b. External seminal vesicle elongated, more than 2.5 x longer than wide 8

8a. Genital atrium shorter than length of ventral sucker; prostatic complex very well-developed with numerous elongated cells.....

.....Gen. n. 2 *magnisaccus* (Machida, 1996)

b. Genital atrium of longer than ventral sucker; prostatic complex not as well-developed as above.....Gen. n. 2 sp. n. 3

CHAPTER VI

ON THE SYSTEMATICS OF SOME MARINE HAPLOPOROIDS (TREMATODA) WITH THE DESCRIPTION OF A NEW SPECIES OF *MEAGASOLENA* LINTON, 1910.

Abstract

Megasolena sp. n. 1 is described from the queen angel, *Holacanthus ciliaris* (Linnaeus) off Florida. The new species can be differentiated from the five other species of *Megasolena* in possessing testes that are smaller in diameter than the ovary. Molecular data are provided for the first time for *Isorchis* cf. *parvus* Durio et Manter, 1969, *Cadenatella americana* Manter, 1937, *Cadenatella floridae* Overstreet, 1969, *Hapladena* cf. *varia*, *Hapladena acanthuri* Siddiqi et Cable, 1960, *Megasolena hysterospina* (Manter, 1931), and *Megasolena* sp. n. 1. Bayesian inference (BI) analysis of partial 28S rDNA sequences of those eight taxa and 45 other haploporoids revealed 1) the Atractotrematidae Yamaguti, 1939 as monophyletic and sister to the rest of the haploporoids tested; 2) the Megasoleninae Manter, 1935 as unresolved with *Hapladena* Linton, 1910 and *Megasolena* Linton, 1910 forming a polytomy; 3) the Cadenatellinae Gibson et Bray, 1982 as monophyletic and basal to the 'mugilid' haploporids; 4) the 'mugilid' haploporids(members of Chalcinotrematinae Overstreet et Curran, 2005, Forticulcitinae Blasco-Costa, Balbuena, Kostadinova et Olson, 2009, Haploporinae Nicoll, 1914, and Waretrematinae Srivastava, 1937) formed a monophyletic clade. A BI analysis of combined Internal Transcribed Spacer Region 2 and partial 28S rDNA sequences revealed a similar topology but

resolved *Megasolena* as basal to the rest of the non-atractotrematid haploporoids. Based on the positions of *Cadenatella*, *Hapladena*, and *Megasolena* relative to the ‘mugilid’ haploporids and the paraphyly of the Megasoleninae, the Haploporidae Nicoll, 1914 requires revision. Therefore, I elevate Cadenatellinae Gibson et Bray, 1982 to Cadenatellidae Gibson et Bray, 1982; elevate the Megasoleninae to the Megasolenidae Manter, 1935; restrict the Haploporidae to the subfamilies Chalcinotrematinae, Forticulcitinae, Haploporidae, and Waretrematinae; and erect Fam. n. 1. I consider the Megasolenidae to include *Megasolena*, *Vitellibaculum* Montgomery, 1957, and *Metamegasolena* Yamaguti, 1970. I consider *Hapladena* Linton, 1910 to be the type genus of Fam. n. 1 and include *Myodera* Montgomery, 1957 in the family.

Introduction

Jones (2005) considered the Haploporoidea Nicoll, 1914 to be comprised of the Atractotrematidae Yamaguti, 1939 and the Haploporidae Nicoll, 1914. Members of the superfamily utilize the alimentary tract or gall bladder of marine (Atractotrematidae and Haploporidae), estuarine (Atractotrematidae and Haploporidae), and freshwater (Haploporidae) herbivorous and omnivorous fishes (e.g., Overstreet and Curran 2005a, b; Bray et al. 2014). Members of the trematode superfamily are morphologically united by the presence of a hermaphroditic sac enclosing the terminal portion of the male and female reproductive structures. Olson et al. (2003) transferred both families into the superfamily Gorgoderoidea Looss, 1901 based on molecular analysis of 18S and 28S rDNA sequences, but remarked that the two families were among the most

labile. Curran et al. (2006) utilized the analysis of 28S rDNA sequences to reinstate the Haploporoidea, and Bray et al. (2014) used the same gene region to demonstrate that *Cadenatella* Dollfus, 1946 (previously attributed to the Enenteridae Yamaguti, 1958) belonged within the superfamily, which they used the subfamily name Cadenatellinae Gibson et Bray, 1982. Members of *Cadenatella* lack a hermaphroditic sac but possess a single testis and utilize herbivorous and omnivorous fishes in the family Kyphosidae Gill. Therefore, Bray et al. (2014) suggested "the terminal genitalia of *Cadenatella* are derived from the hermaphroditic sac by loss of the wall" (20).

Atractotrematidae has been considered a junior synonym of the Haploporidae (Durio and Manter 1969, Ahmad 1985). However, that view has not prevailed on a morphological (e.g., Yamaguti 1971, Overstreet and Curran 2005a, b) basis or by molecular analysis (e.g., Blasco-Costa et al. 2009, Pulis and Overstreet, 2013, Andres et al. 2014a). However, Overstreet and Curran (2005b) considered the status of the Atractotrematidae as tentative, as the family is depauperate and has yet to have a lifecycle published. Additionally, molecular data have not been provided for any atractotrematid taxon since the study by Olson et al. (2003), but those for the haploporids have (Blasco-Costa et al. 2009, Blasco-Costa et al. 2010, Pulis and Overstreet 2013, Pulis et al. 2013, Bray et al. 2014, Besprozvannykh et al. 2014, Pulis, 2014, Andres et al. 2014a, Chapters III-V).

Linton (1910) erected *Megasolena* Linton, 1910 for *Megasolena estrix* Linton, 1910 from the Bermuda sea chub, *Kyphosus sectatrix* (Linnaeus) and

Hapladena Linton, 1910 for *Hapladena varia* Linton, 1910 from the doctorfish *Acanthurus chirurgus* (Bloch) (as *Teuthis hepatus* non Linnaeus). Manter (1935) redescribed *M*. *estrix* and erected the Megasoleninae for *Megasolena* and *Hapladena* and placed them within the Opistholebetidae Fukui, 1929 based on possessing a lymphatic system and 'muscular bulb around the prepharynx'. Manter (1940) discussed the likely close affinity of *Carassotrema* Park, 1938 with *Hapladena* based on a single testis and a hermaphroditic sac, and stated that the "presence or absence of lymphatic vessels may not be of such great significance" (345) in the systematics of the megasolenines. Skrjabin (1942) erected the Megasolenidae Skrjabin, 1942 for *Megasolena*, *Hapladena*, and *Carassotrema*, and Yamaguti (1942) also erected the Megasolenidae Yamaguti, 1942 for the same genera; however, both authorities were incorrectly applied, and the name should be Megasolenidae Manter, 1935 (Article 36, *International Code of Zoological Nomenclature*). Subsequently, Yamaguti (1953, 1958, 1971) considered the Megasoleninae as a subfamily of the Waretrematidae Srivastava, 1937, even though others considered the Waretrematinae and Megasolenidae as synonyms of Haploporidae (e.g., Manter 1957, Siddiqi and Cable 1960, Manter and Pritchard 1961). Nasir and Gómez (1976) provide a review of the interrelationships of the Haploporidae up until that time.

Overstreet and Curran (2005b) reviewed the Haploporidae Looss, 1902 and accepted four subfamilies the Haploporinae Nicoll, 1914; the Megasoleninae Manter, 1935 (syn. Scorpidicolinae Yamaguti, 1971); the Waretrematinae Srivastava, 1937; and erected the Chalcinotrematinae Overstreet et Curran,

2005. Blasco-Costa et al. 2009a erected a fifth subfamily, the Fotriculcitinae Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009, from mugilids based on morphological and molecular data. Within the Megasoleninae Overstreet and Curran (2005a) accepted *Megasolena* Linton, 1910; *Hapladena* Linton, 1910 (syns. *Deredena* Linton, 1910; *Hairana* Nagaty, 1948); *Vitellibaculum* Montgomery, 1957 (syn. *Allomegasolena* Siddiqi et Cable, 1960); *Myodera* Montgomery, 1957 (syn. *Scorpidicola* Montgomery, 1957); and *Metamegasolena* Yamaguti, 1970; however, they suggested that the subfamily may be polyphyletic. Overstreet and Curran (2005a) suggested that the genera containing members with two testes (*Megasolena*, *Metamegasolena*, and *Vitellibaculum*) may not belong within the Haploporidae since the rest of the haploporids have a single testis. Presumably, they believed that those genera if not belonging within the Haploporidae, may have a closer affiliation with the Atractotrematidae. Atractotrematids possess a hermaphroditic sac and two testes, but they generally have a smaller, circular to fusiform body and oblique testes rather than tandem to semi-oblique testes. The convoluted taxonomic history of the Megasoleninae, the revelation of the Cadenatellinae within the Haploporoidea, and the poor molecular representation of the Atractotrematidae, Cadenatellinae, and Megasoleninae compared with the ‘mugilid’ haploporids (members of the Haploporidae, Chalcinotrematinae, Forticulcitinae, and Waretrematidae) illustrate the need for increased molecular representation of those three groups. The purpose of this study is to help clarify some of the

deeper relationships of the Haploporoidea and determine the validity of the Haploporidae (sensu Overstreet and Curran 2005a).

Materials and Methods

During February 2010 specimens of *Isorchis* cf. *parvus* were collected from the milkfish, *Chanos chanos* (Forsskål), by a cast-net from off Learmouth, Western Australia, Australia. Specimens of *Cadenatella americana* Manter, 1949 and *Cadenatella floridae* Overstreet, 1969 were recovered from specimens of *K. sectatrix* off Long Key, Florida, USA, by baited hook-and-line in July 2012. During April 2009 dead specimens of *Hapladena* were recovered from specimens of the doctorfish, *A. chirurgus*, purchased at a fish market in Christiansted, St. Croix, U.S. Virgin Islands. Specimens of *Megasolena hysterospina* were obtained by Eric Pulis and me from the Western Atlantic seabream, *Archosargus rhomboidalis* (Linnaeus), off Missouri Key and Marathon, Florida, USA, in November 2011 and July, 2012, and by Robin Overstreet from the Bermuda porgy, *Diplodus bermudensis* Caldwell, from Harrington Sound, Bermuda. Specific fish names follow those given by FishBase (Froese and Pauly 2014). Haploporoids were isolated following the method of Cribb and Bray (2010) for gastrointestinal species but skipping the initial examination under a dissecting microscope because of the large volume of intestinal contents. The worms were rinsed and cleaned in a container with saline and examined briefly; then, most of the saline was decanted, the worms were killed by pouring hot (not boiling) water over them, and they were fixed in 70% ethanol. Worms were stained in Mayer's haematoxylin, dehydrated in a graded ethanol series, cleared in methyl

salicylate, and mounted permanently in Dammar gum. Measurements were made using a compound microscope equipped with a differential interference contrast, a Canon EOS Rebel T1i camera, and calibrated digital software (iSolutions Lite ©). All measurements are in micrometres; for descriptions of new species, data for the type specimen are followed by the range of data for the other specimens in parenthesis, and for reports of other species supplemental data are provided. Terminology of the hermaphroditic sac and its structures follows the terms used by Pulis and Overstreet (2013). Museum abbreviations are as follows: QM, Queensland Museum, Brisbane, Queensland, Australia; USNM, Smithsonian National Museum of Natural History, Washington, DC, USA; and USNPC, United States National Parasite Collection (previously in Beltsville, Maryland, USA and now at USNM). Representative specimens will be submitted to museums before the chapter is submitted for publication, thus collection numbers for new material are listed as to be determined (TBD).

Genomic DNA was extracted from one hologenophore sensu Pleijel et al. (2008) of the new species and *C. americana*, two hologenophores of *C. floridae*, and three entire specimens of *I.* cf. *parvus* and *M. hysterospina*, either fixed in cool 95% ethanol or heat killed worms in 70% ethanol using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the instructions provided. DNA fragments ca 2,400 base pairs (bp) long, comprising the 3' end of the 18S nuclear rRNA gene, internal transcribed spacer region (including ITS1 + 5.8S + ITS2), and the 5' end of the 28S rRNA gene (including variable domains D1–D3), were amplified from the extracted DNA by polymerase chain reaction

(PCR) on a PTC-200 Peltier Thermal Cycler using forward primer ITSF (5'-CGCCCGTCGCTACTACCGATTG-3') and reverse primer 1500R (5'-GCTATCCTGAGGGAAACTTCG-3'). These PCR primers and multiple internal primers were used in sequencing reactions. The internal forward primers were DIGL2 (5'-AAGCATATCACTAAGCGG-3'), 300F (5'-CAAGTACCGTGAGGGAAAGTTG-3'), and 900F (5'-CCGTCTTGAAACACGGACCAAG-3') and the internal reverse primers were 300R (5'-CAACTTTCCCTCACGGTACTTG-3'), DIGL2R (5'-CCGCTTAGTGATATGCTT-3'), and ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'). The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences from the species were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 4.10.1) and submitted to GenBank. Sequences of the partial 28S rRNA gene (henceforth referred to as 28S) and ITS2 rRNA gene (henceforth referred to as ITS2) were obtained from GenBank are as follows: *Atractotrema sigani* Durio et Manter,1969 (AY222267) (Olson et al. 2003), *Cadenatella isuzumi* Machida, 1993 (FJ788497) (Bray et al. 2009), *Cadenatella pacifica* (Yamaguti, 1970) (FJ788498) (Bray et al. 2009), *Capitimitta costata* Pulis et Overstreet, 2013 (KC206497) (Pulis and Overstreet 2013), *Capitimitta darwinensis* Pulis et Overstreet, 2013 (KC206498) (Pulis and

Overstreet 2013), *Capitimitta* sp. (KC206499) of Pulis and Overstreet (2013), *Dicrogaster contracta* Looss, 1902 (FJ211262; ITS2 FJ211268) (Blasco-Costa et al. 2009a), *Dicrogaster perpusilla* Looss, 1902 (FJ211238; ITS2 FJ211248) (Blasco-Costa et al. 2009a), *Forticulcita gibsoni* Blasco-Costa, Montero, Balbuena, Raga, et Kostadinova, 2009 (FJ211239; ITS2 FJ211234) (Blasco-Costa et al. 2009a), *Hapladena nasonis* Yamaguti, 1970 (AY222265) (Olson et al. 2003), *Haploporus benedeni* Looss, 1902 (FJ211237; ITS2 FJ211247) (Blasco-Costa et al. 2009a), *Intromugil alachuaensis* Pulis, Fayton, Curran, et Overstreet, 2013 (KC430095) (Pulis et al. 2013), *Intromugil mugilicolus* (Shireman, 1964) (KC430096) (Pulis et al. 2013), *Lecithobotrys putrescens* Looss, 1902 (FJ211236; ITS2 FJ211246) (Blasco-Costa et al. 2009a), *Litosaccus brisbanensis* (KM253765) (Andres et al. 2014a), *Paragonimus kellicotti* Ward, 1908 (HQ900670) (Fischer et al. 2011), *Paragonimus westermani* (Kerber, 1878) (AY116874) (Olson et al. 2003), *Parasaccocoelium haematocheilum* Besprozvannykh, Atopkin, Ermolenko, et Nikitenko, 2014 (HF548461) (Besprozvannykh et al. 2014), *Parasaccocoelium mugili* Zhukov, 1971 (HF548468) (Besprozvannykh et al. 2014), *Parasaccocoelium polyovum* (HF548474) (Besprozvannykh et al. 2014), *Pseudomegasolena ishigakiense* Machida et Kamiya, 1976 (AY222266) (Olson et al. 2003), *Saccocoelioides* sp. of Curran et al. (2006) (EF032696), *Saccocoelium brayi* Blasco-Costa, Montero, Balbuena, Raga, Kostadinova, et Olson, 2009 (FJ211234; ITS2 FJ211244) (Blasco-Costa et al. 2009a), *Saccocoelium cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga, et Kostadinova, 2009 (FJ211233; ITS2 FJ211243)

(Blasco-Costa et al. 2009a), *Saccocoelium obesum* Looss, 1902 (FJ211260; ITS2 FJ211265) (Blasco-Costa et al. 2009a), *Saccocoelium tensum* Looss, 1902 (FJ211258; ITS2 FJ211263) (Blasco-Costa et al. 2009a), and *Spiritestis herveyensis* Pulis et Overstreet, 2013 (KC206500) (Pulis and Overstreet 2013). Sequences of *Forticulcita* sp. n. 1 (SSC23), *Forticulcita* sp. n. 2 (SJ3-1), and Gen. n. 1 *fastigata* (MJA281) from Chapter III; *Pseudodicrogaster* sp. n. 1, *Pseudodicrogaster* sp. n. 2, and *Pseudodicrogaster* sp. n. 3 from Chapter IV; Gen. n. 2 sp. n. 1 (MJA303), Gen. n. 2 sp. n. 2 (EP561), Gen. n. 2 sp. n. 3 (EP165), Gen. n. 2 sp. n. 4 (EP154), Gen. n. 2 *magnisaccus* (Machida, 1996) (MJA769), Gen. n. 2 *megasacculum* (Liu, Wang, Peng, Yu, et Yang, 2004), and Gen. n. 2 *mugilis* (Liu et Yang, 2002) from Chapter V; and sequences of *Carassotrema estuarinum* Tang et Lin, 1979 (EP198), *Hurleytrematoides chaetodoni* (Manter, 1942) (MJA110), *Lasiotocus haemuli* Overstreet, 1969 (MJA608), *Malabarotrema lobolecithum* (Martin, 1973) (EP568), *Malabarotrema megaorchis* Liu et Yang, 2002 (EP644), *Malabarotrema* sp. 1 (EP148), *Saccocoelioides* sp. (EP344), *Unisaccoides vitellosus* Martin, 1973 (EP379), *Unisaccoides* sp. 1 (EP077), *Unisaccus brisbanensis* Martin, 1973 (EP376), *Unisaccus lizae* (Liu, 2002) (EP640), *Unisaccus* sp. 1 (EP227), and *Unisaccus* sp. 2 (EP591) from Pulis (2014) are also used. The sequences were aligned using MAFFT version 6.611b (Katoh et al. 2005) with 1,000 cycles of iterative refinement and the *genafpair* algorithm. The alignment was masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded and the alignment was trimmed to the shortest sequence on both

5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999). The resulting 28S alignment utilized 2 species of *Paragonimus* and 55 haploporoids with *P*. *westermani* as the outgroup based on its phylogenetic position relative to the Haploporoidea (Olson et al. 2003) and to be consistent with previous analyses (Pulis et al. 2013, Bray et al. 2014, Andres et al. 2014a). The resulting assay combined ITS2 and 28S alignment utilized two monorchiids and 50 haploporids with *Hurleytrematoides chaetodoni* (Manter, 1942) as the outgroup based on its phylogenetic position relative to the Haploporoidea (Olson et al. 2003) and because of the unreliability of the ITS2 sequences of the two species of *Paragonimus* Braun, 1989 available on GenBank. Phylogenetic analyses of the data were performed using BI with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The best nucleotide substitution model was estimated with jModeltest-2 (Darriba et al. 2012) as general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ) for the 28S and GTR + Γ for the ITS2 regions. The following model parameters were used in MrBayes for the 28S only analysis: nst = 6, rates = invgamma, ngen = 1,000,000 and samplefreq = 100. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al. 2001) with all other settings left as default. For the combined ITS2 and 28S analysis, the model was partitioned and run with the same parameters.

All pairwise comparisons of sequence data exclude gaps. Pairwise comparison of newly generated 28S sequences and those obtained from GenBank were trimmed to the shortest sequence.

Results

Molecular analysis

The DNA sequence fragment lengths for the newly provided specimens are in Table 6.1. No intraspecific variation was observed in any of the sequences when multiple specimens were sequenced. Sequences of *C. americana* and *C. floridae* did not differ in the 3' end of 18S, differed by 49 bp (9.6%) in the ITS1, differed by 1 bp (0.6%) in the 5.8S, 25 bp (9.8%) in the ITS2, and 45 bp (3.3%) in the 28S. Sequences of *Helicometra* cf. *varia* and *H*. *acanthuri* differed by 2 bp (2.2%) in the 3' end of the 18S, by 23 bp (3.8%) in the ITS1, 16 bp (5.7%) in the ITS2, and 32 bp (2.3%) in the 28S, and did not differ in the 5.8S. Sequences of *M. hysterospina* and *Megasolena* sp. n. 1 did not differ in either the 3' end of the 18S nor in the 5.8S; and they differed by 6 bp (1.1%) in the ITS1, 1 bp (0.3%) in the ITS2, and 9 bp (0.7%) in the 28S. Pairwise comparisons of 28S sequence data of *Isorchis* cf. *parvus* with the two atractotrematids from GenBank are in Table 6.2. Pairwise comparisons of 28S sequence data of the two species of *Cadenatella* I generated with the two species of *Cadenatella* from GenBank are in Table 6.3. Pairwise comparisons of 28S sequence data of the two species of *Hapladena* I generated with *H. nasonis* from GenBank are in Table 6.4.

The 28S alignment utilized two species of *Paragonimus* and 55 haploporoids and was 1,147 characters long with 605 conserved sites, 542

variable sites, and 456 informative sites. The 28S BI analysis used the two species of *Paragonimus* as the outgroup and an ingroup containing 55 haploporoids (Figure 6.1). The Atractotrematidae was revealed as monophyletic and sister to the rest of the haploporoids. The Haploporidae was revealed as paraphyletic, as was demonstrated by previous authors (Bray et al. 2014, Andres et al. 2014a, Chapter III-V), with *Hapladena* and *Megasolena* forming a polytomy sister to *Cadenatella*. *Cadenatella* was sister to the 'mugilid' haploporids. The 'mugilid' haploporids formed two main subclades: 1) Waretrematinae and 2) Forticulcitinae + Chalcinotrematinae and Haploporinae.

Table 6.1

Base pair lengths of sequences for the partial 18S rDNA, ITS1, 5.8S rDNA, ITS2, and partial 28S of Isorchis cf. parvus, Cadenatella americana, C. floridae, Megasolena sp. n. 1, M. hysterospina, Hapladena acanthuri, and H. cf varia.

	18S	ITS1	5.8S	ITS2	28S
Isorchis cf. *parvus*	114	514	157	265	1,393
Cadenatella americana	107	511	157	258	1,384
Cadenatella floridae	107	517	157	258	1,384
Megasolena sp. n. 1	101	552	157	294	1,392
Megasolena hysterospina	101	551	157	294	1,392
Hapladena acanthuri	89	602	157	279	1,407
Hapladena cf *varia*	89	603	157	279	1,407

Table 6.2

Pairwise comparisons (trimmed to GenBank sequence no AY222266; excluding gaps) of percent nucleotide similarity and number of base pair differences (in parentheses) of the partial 28S sequences of the three atractotrematids.

	Atractotrema sigani AY222266	*Isorchis* cf. *parvus*
Isorchis cf. *parvus*	89.7 (129)	-
Pseudomegasolena ishigakiensis AY222266	88.9 (138)	96.7 (41)

Table 6.3

Pairwise comparisons (trimmed to GenBank sequences; excluding gaps) of percent nucleotide similarity and number of base pair differences (in parentheses) of the partial 28S sequences of the four species of Cadenatella.

	Cadenatella pacifica FJ788498	*Cadenatella americana*	*Cadenatella floridae*
Cadenatella americana	92.6 (98)	-	-
Cadenatella floridae	92.1 (105)	96.7 (44)	-
Cadenatella isuzumi FJ788497	88.7 (150)	89.4 (141)	88.7 (151)

Table 6.4

Pairwise comparisons (trimmed to GenBank sequence no AY222265; excluding gaps) of percent nucleotide similarity and number of base pair differences (in parentheses) of the partial 28S sequences of the three species of Hapladena.

	Hapladena nasonis AY222265	*Hapladena acanthuri*
Hapladena acanthuri	92.2 (101)	-
Hapladena cf *varia*	91.5 (110)	97.5 (32)

The combined ITS2 and 28S alignment utilized 2 monorchiids and 50 haploporoids. The ITS2 alignment was 262 characters long with 75 conserved sites, 187 variable sites, and 162 informative sites and the 28S was 1,142 characters long with 618 conserved sites, 524 variable sites, and 441 informative sites. The BI analysis used *H*. *chaetodoni* as the outgroup and an ingroup containing 50 haploporoids (Figure 6.2). The combined dataset resolved a similar topology with the only atractotrematid, *I*. cf. *parvus*, as sister to all the other taxa and *Cadenatella* as sister to the 'mugilid' haploporids. The combined analysis resolved the ‘megasolenine’ polytomy, with *Megasolena* as sister to *Hapladena* +

Cadenatella and the ‘mugilid’ haploporids. The only other difference was in the interrelationships of the 'mugilid' haploporids that also formed two main subclades but with: 1) Haploporinae and 2) a polytomy consisting of the Forticulcitinae + *Saccocoelioides nannii* +*Intromugil* spp.+ *Spiritestis herveyensis* + the rest of the Waretrematinae.

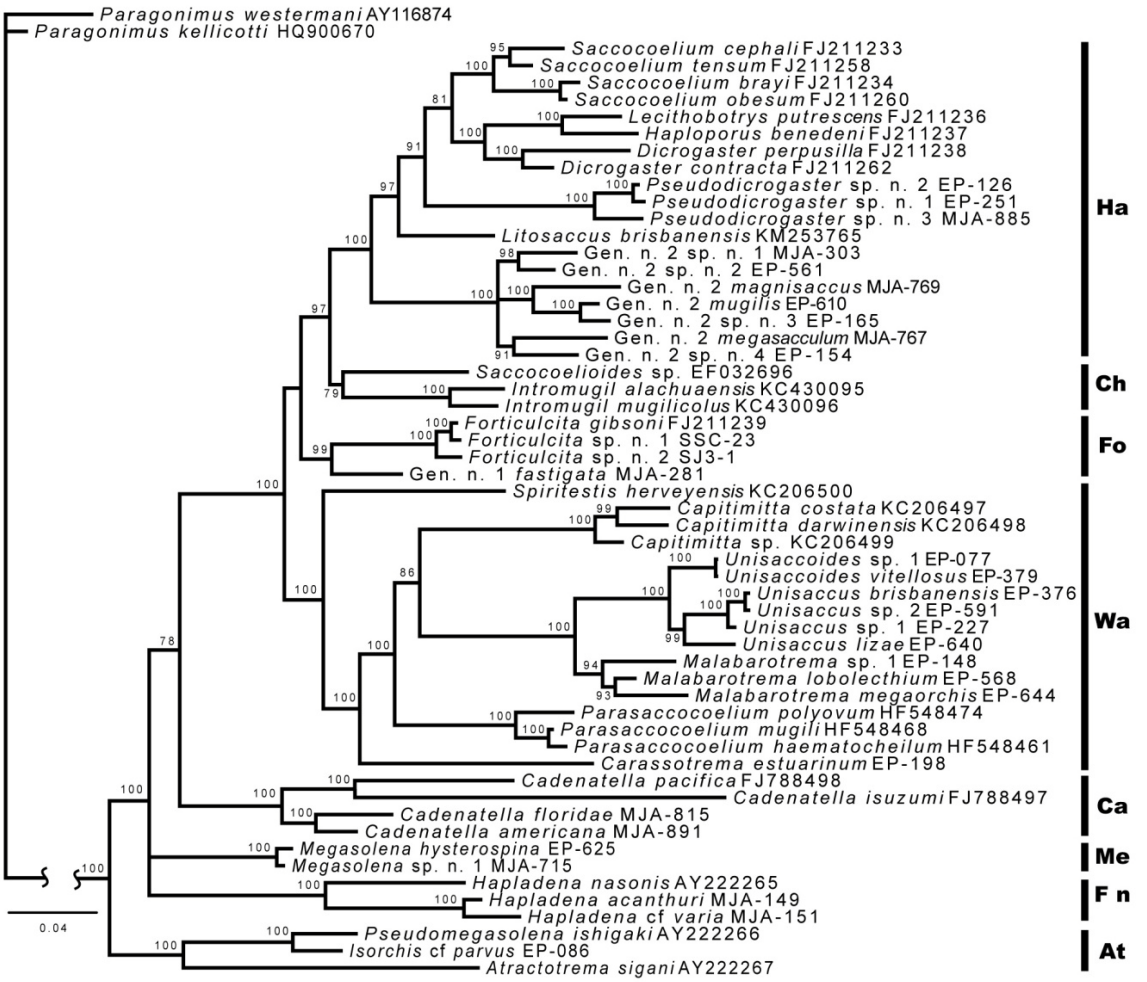


Figure 6.1. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100) demonstrating that the Megasoleninae is paraphyletic. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellidae; Ch = Chalcinotrematinae; F n = Fam. n. 1; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasolenidae; Wa = Waretrematinae.

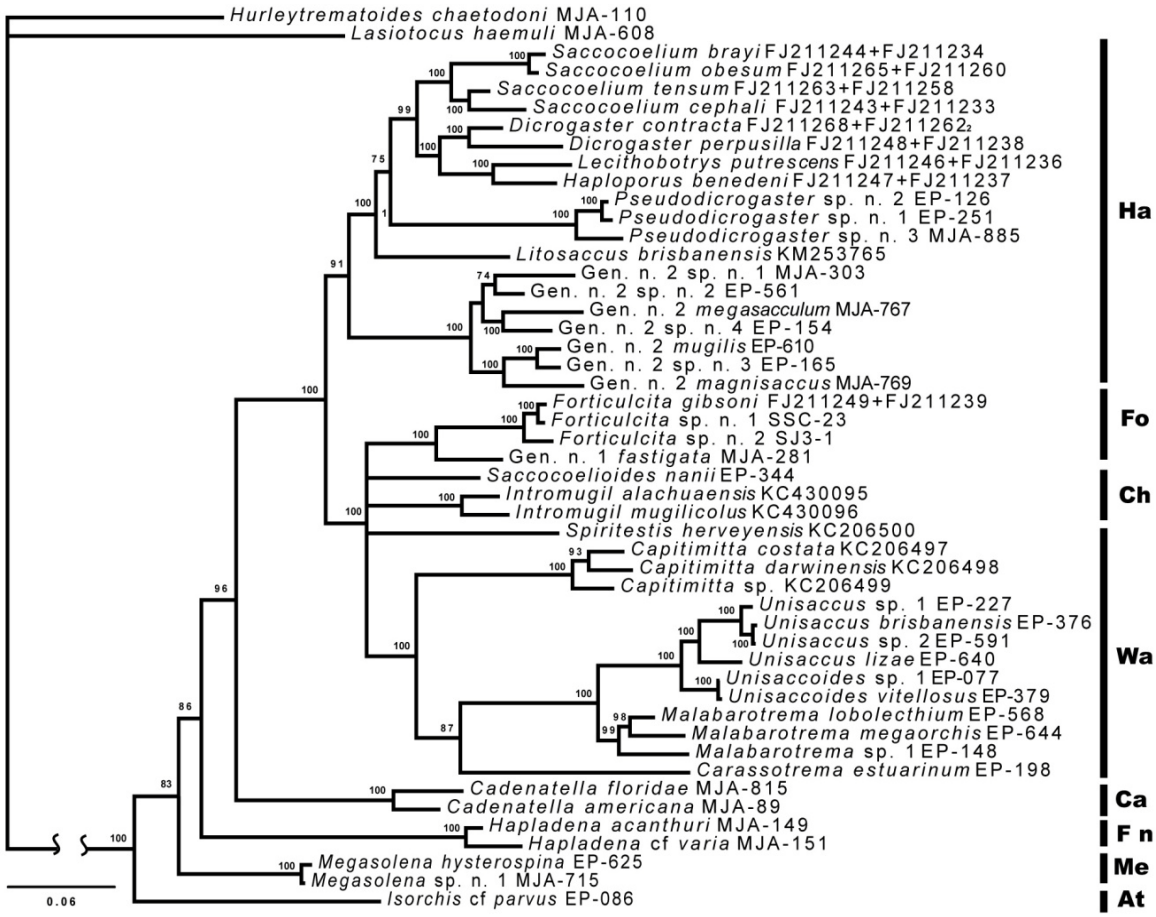


Figure 6.2. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 28S rDNA and internal transcribed spacer region 2 (ITS2) sequences (Model parameters: GTR + I + Γ for the 28S partition; GTR + Γ for the ITS2 partition;1,000,000 generations and a sample frequency of 100) demonstrating that the Megasoleninae is paraphyletic. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellidae; Ch = Chalcinotrematinae; F n = Fam. n. 1; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasolenidae; Wa = Waretrematinae.

Based on my phylogenetic analyses the Haploporidae is restricted to the members of Haploporinae, Chalcinotrematinae, Forticulcitinae, and Waretrematinae. I elevate Cadenatellinae and Megasolinae to the rank of family, and erect a new family for *Hapladena*.

Atractotrematidae Yamaguti, 1939

Isorchis cf. parvus Durio et Manter, 1969

Type and only known host: *Chanos chanos* (Forsskål), milkfish, Chanidae.

Type-locality: off New Caledonia.

Other locality: off Learmonth, Western Australia, Australia (22°12'41"N, 114°5'59"E).

Site: Intestine.

Holotype: USNPC 63319.

Supplemental material: Western Australian Museum, Perth, Western Australia, Australia TBD, QM TBD, USNM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP-86) from 3 entire specimens.

Remarks. *Isorchis* Durio et Manter, 1969 was established by Durio and Manter (1969) for *I*. *parvus* from *Chanos chanos* off New Caledonia. They stated that *Isorchis* "includes the type species, *I*. *parvus*, and an undescribed species collected in Australia," (296) but did not provide a description for the Australian species. Thus, although my specimens of *Isorchis* morphologically agree with the description of *I*. *parvus*, I am not positive that they are the same species but do not have material necessary to make a formal description for my specimen. Particularly, as the two other species of *Isorchis* also occur in *C*. *chanos*.

Cadenatellidae Gibson et Bray, 1982

Diagnosis. Body elongate. Tegument spined. Oral sucker with 8-10 lobes on dorsal and dorsolateral margins. Ventral sucker approximately in anterior third to eighth of body length. Pharynx well-developed. Prepharynx shorter to longer than pharynx. Oesophagus variable. Intestinal bifurcation anterior to ventral sucker. Uroproct present. Testis single, elongate, ellipsoidal to irregular, in anterior to mid-hindbody. Hermaphroditic sac absent. Seminal vesicle sinuous, extending into hindbody. Pars prostatica vesicular, short. Hermaphroditic duct short. Genital pore median, anterior to ventral sucker, with one or more accessory suckers. Ovary entire, rounded to oval, pretesticular. Uterus confined between ovary and hermaphroditic duct. Uterine seminal receptacle present. Eggs thin-shelled. Vitellarium with numerous small follicles, restricted to hindbody. Excretory vesicle reaches ovary or ventral sucker. In marine teleosts (*Kyphosus*).

Type-genus: *Cadenatella* Dollfus, 1946.

Remarks. *Cadenatella* was originally allocated to the Enenteridae but was resolved as a haploporioid using BI analysis of partial 28S rDNA sequences (Bray et al. 2014). They remarked that the Cadenatellinae will likely be recognized at the family level once additional molecular data for members of the Haploporioidea were available. My molecular analysis revealed *Cadenatella* as monophyletic and occupying a position on the tree that has been consistent with recent molecular analyses (Andres et al. 2014a, Chapters III-V). In the diagnosis, I use the term 'hermaphroditic duct' rather than genital atrium as the structure

has been previously called. The cadenatellids are the only currently known haploporoids that lack a hermaphroditic sac surrounding the terminal genitalia. Thus, their inclusion in the Haploporioidea strongly suggests a secondary loss of the wall of the hermaphroditic sac (Bray et al. 2014) and that at least a portion of this structure is a hermaphroditic duct. The Cadenatellidae is distinguished from all other Haploporoidea by its members lacking a hermaphroditic sac.

Cadenatella americana Manter, 1949

Type-host: *Kyphosus incisor* (Cuvier), yellow sea chub, Kyphosidae.

Other host: *Kyphosus sectatrix* (Linnaeus), Bermuda sea chub, Kyphosidae.

Type-locality: off Tortugas, Florida, USA.

Other localities: Biscayne Bay, Florida; off Long Key, Florida (24°50'22"N, 80°46'48"W).

Site: Intestine.

Holotype: USNPC 46365.

Supplemental material: Body 1,953-2,968 long, 271-398 wide at level of final third of BL representing 10-18% of BL. Forebody 471-774 long representing 24-26% of body length (BL). Hindbody 1,298-1,995 long representing 66-68% of BL. Oral sucker 206-225 long, 199-232 wide. Ratio of oral sucker width to ventral sucker widths 1: 0.7-0.8. Prepharynx 142-227. Testis 393-555 long, 152-234 wide. Posttesticular field 600-1,060 representing 30-36% of BL. Ovary 108-118 long, 86-138 wide; 238-367 from posterior margin of ventral sucker, contiguous with testis. Eggs 45-52 long, 31-38 wide. USNM TBD, QM TBD; Figure 6.3A.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-891) from 1 hologenophore.

Remarks. This species was described by Manter (1949) as having a retracted oral sucker, contracted forebody, and lateral outfoldings of the proximal portion of the prepharynx that likely resulted from contraction of the body. My specimens all have the oral sucker protruded and a distinct prepharynx, thus the features that Manter (1949) reported are the result of contraction of his specimen. Manter (1949) also reported a lateral indentation in the testis of his specimen that was not observed in mine. He also reported a small, inconspicuous, thin-walled cirrus sac. Overstreet (1969) stated that "a thin membrane appears to surround the vesicle in some wholemounts" (134), a feature I did not observe in my specimens.

Cadenatella floridae Overstreet, 1969

Type-host: *Kyphosus sectatrix* (Linnaeus), Bermuda sea chub, Kyphosidae.

Type-locality: Biscayne Bay, Florida, USA.

Other localities: off Long Key, Florida, USA (24°50'22"N, 80°46'48"W).

Site: Pyloric caeca and intestine.

Holotype: USNPC 71367.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-815) from 2 hologenophores.

Supplemental material: USNM TBD, QM TBD; Figure 6.3B.

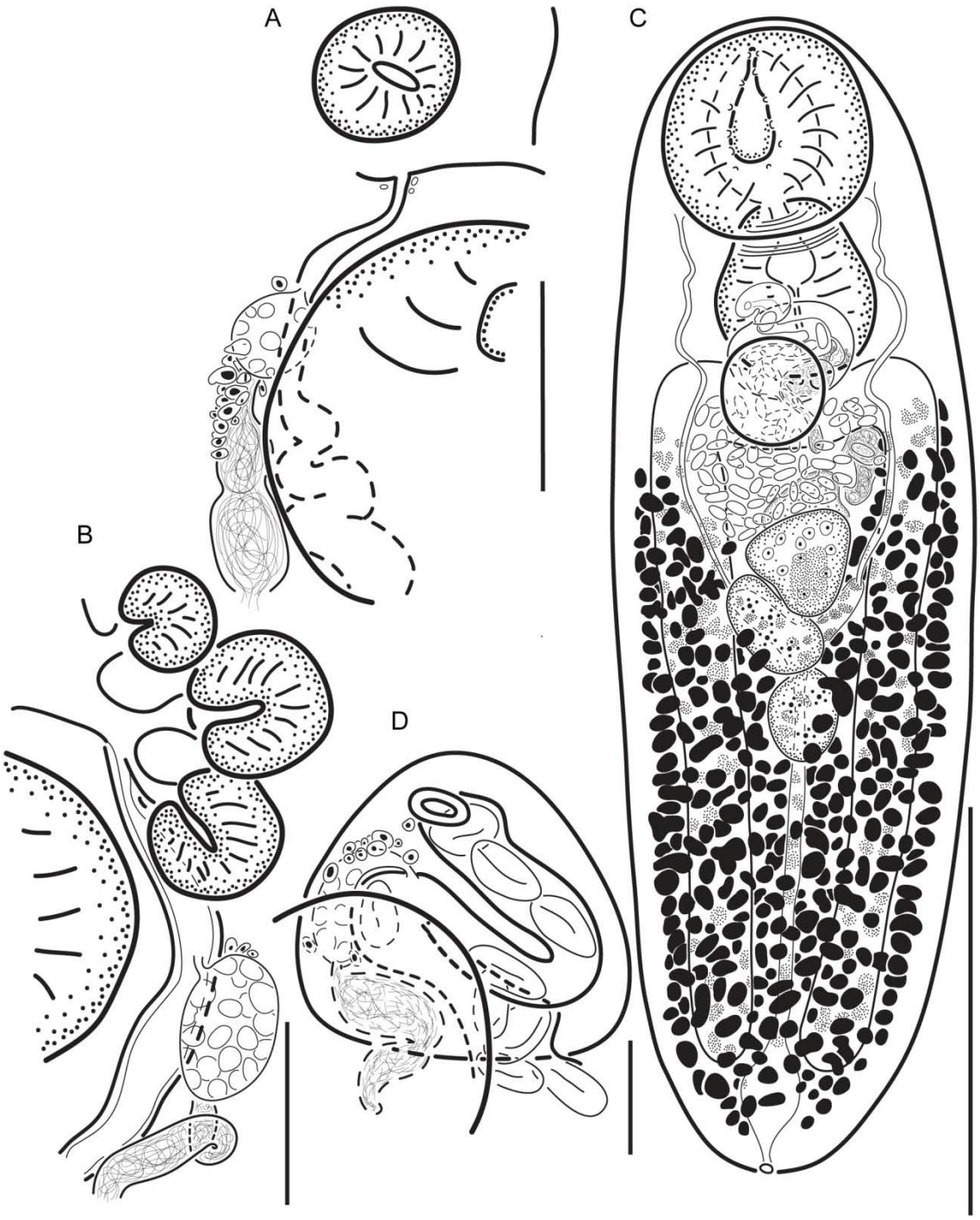


Figure 6.3. A. Terminal genetalia of *Cadenatella americana*. B. Terminal genetalia of *Cadenatella floridae*. C-D. *Megasolena* sp. n. 1. C. Ventral view of holotype with everted hermaphroditic sac. D. Ventral view of hermaphroditic sac. Scale bars: A-B, D 100µm; C 1,000µm.

Megasolenidae Manter, 1937

Diagnosis. Body fusiform to elongate. Tegument thick, spined or unspined. Eyespot pigmentation absent to diffuse. Oral sucker subterminal to terminal. Ventral sucker near anterior third of body. Prepharynx variable. Pharynx large, well-developed. Oesophagus variable. Caeca sac-like to cylindrical, terminating blindly at level of posterior third of hindbody to near posterior margin of body. Testes 2, tandem to slightly oblique, contiguous to separated. Hermaphroditic sac subglobular to elongate. External seminal vesicle elongate, narrow. Ovary immediately pretesticular. Uterus confined between ovary and hermaphroditic sac. Eggs non-operculate, non-filamented. Vitellarium with numerous small, large, or dendritic follicles, filling available space or lateral fields of hindbody. Lymphatic system present. Excretory vesicle I- or Y-shaped. In marine fishes.

Type-genus: *Megasolena* Linton, 1910.

Remarks. I consider *Megasolena*, *Metamegasolena*, and *Vitellibaculum* to belong in Megasolenidae. Overstreet and Curran (2005a) suggested that those three genera may not belong in the Haploporidae, as they possess two testes rather than a single testis. The family can be differentiated from the other haploporoids except the Atractotrematidae in processing two testes. Megasolenidae can be differentiated from the Atractotrematidae in possessing tandem to slightly oblique testes rather than oblique testes and in having a larger body and more robust tegument.

Megasolena hysterospina (Manter, 1931) Overstreet, 1969

syns. *Lepidauchen hysterospina* Manter, 1931; *Megasolena archosargi* Sogandares-Bernal et Hutton, 1959.

Type-host: *Lagodon rhomboides* (Linnaeus), pinfish, Sparidae.

*Other host*s: *Archosargus probatocephalus* (Walbaum), sheepshead; *Archosargus rhomboidalis* (Linnaeus), Western Atlantic seabream; *Diplodus bermudensis* Caldwell, Bermuda porgy, all Sparidae.

Type-locality: off Beaufort, North Carolina, USA.

Other localities: Bayboro Harbor, Tampa Bay, Florida, USA; Biscayne Bay, Florida; Little Duck Key, Florida (24°40'47"N, 81°14'5"W); Marathon, Florida (24°46'31"N, 80°55'46"W); Harrington Sound, Bermuda (32°19'23"N, 64°44'12"W).

Site: Intestine.

Holotype: USNPC 8432.

Material Examined: 6 specimens from off Marathon, Florida; 4 specimens from Harrington Sound, Bermuda.

Supplemental material: USNM TBD, QM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (EP-625) from 1 entire specimen from Harrington Sound, Bermuda; 2 immature specimens from off Marathon, Florida.

Remarks. My specimens collected off Florida and those collected by RMO off Bermuda are similar to those described by Manter (1931) and Overstreet

(1969). I agree with Overstreet (1969) in considering *M. archosargi* a junior synonym of *M*. *hysterospina*, based on morphological features and host similarity.

Megasolena sp. n. 1 Figure 6.3C-D.

Description (measurements based on 5 gravid wholemounts). Body elongate, cylindrical, 2,048 (2,563–3,054) long, 829 (776–970) wide at level of midbody, with width representing 30 (28–33)% of BL. Tegument unspined. Forebody 761 (752–936) long representing 28 (27–31)% of BL. Hindbody 1,715 (1,574–1,835) long representing 63 (60–63)% of BL. Oral sucker subglobular, subterminal, 484 (428–513) long, 500 (461–541) wide, with anterior periphery surrounded by ring of approximately 12 small papillae. Ventral sucker 262 (237–286) long, 245 (233–294) wide. Ratio of oral sucker to ventral sucker widths 1:0.5 (1:0.5–0.5). Prepharynx 34 (57–68) long. Pharynx subglobular, 442 (357–432) long, 377 (316–410) wide. Ratio of oral sucker width to pharynx width 1:0.8 (1:0.7–0.8). Oesophagus 21 (30–67) long. Intestinal bifurcation dorsal to level of ventral sucker. Caeca long, approximately 8–10 times longer than wide, terminating blindly, 206 (224–254) from posterior end, with postcaecal space representing 8 (7–9)% of BL.

Testes contiguous, slightly oblique, irregular, near midbody, intercaecal; anterior testis 231 (167–177) long, 186 (165–186) wide; posterior testis 228 (174–229) long, 182 (152–188) wide. Posttesticular space 967 (809–1,009) long representing 35% (32–34%) of BL. External seminal vesicle elongate, 73 (48–106) long, 12 (21–44) wide, posterior to ventral sucker. Hermaphroditic sac thin-

walled, anterodorsal to dorsal of ventral sucker, 270 (267–327) long, 305 (279–377) wide representing 103% (106–114%) of ventral sucker length, containing internal seminal vesicle 126 (81–140) long by 86 (43–91) wide with short prostatic bulb and with short male duct; female duct, and hermaphroditic duct; male and female ducts unite at approximately midlevel of hermaphroditic sac; hermaphroditic duct muscularised, approximately 1/2 length of hermaphroditic sac. Genital pore medial, 64 (61–86) anterior to anterior margin of ventral sucker.

Ovary irregular to longitudinally elongate, medial, intercaecal, contiguous with anterior testis 244 (204–240) long, 254 (246–344) wide, 174 (179–201) from posterior margin of ventral sucker. Mehlis' gland anterodorsal to ovary, 131 long (103–151), 208 (109–227) wide. Uterus confined between anterior margin of ovary and hermaphroditic sac. Laurer's canal containing sperm, posterosinistral to Mehlis'gland, opening dorsally. Vitellarium follicular, extending from near posterior margin of body to near anterior margin of ventral sucker, surrounding caeca, dorsal to reproductive organs, ventral to testes; vitelline reservoir dorsal to ovary. Eggs thin-shelled, numerous, 72–74 (70–76) long, 32–34 (31–36) wide.

Excretory vesicle Y-shaped, branching near posterior margin of ovary; pore terminal.

Type and only known host: *Holacanthus ciliaris* (Linnaeus), queen angelfish, Pomacanthidae.

Type- locality: West Florida Middle Grounds, Gulf of Mexico (28°32'37"N, 84°46'43"W) approximately 130 km south of Apalachicola, Florida, USA; from 59 m depth.

Site: Intestine.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-735) from 1 entire immature specimen, and 1 hologenophore.

Remarks. *Megasolena* sp. n. 1 can be differentiated from all other members in the genus by possessing an ovary that is larger in diameter than the testis. It can be further differentiated from *Megasolena hysterospina* (Manter, 1931) and *Megasolena dongzhaiensis* Liu, Zhou, Yu et Liu, 2006 in having an aspinous tegument. *Megasolena* sp. n. 1 can be differentiated from *Megasolena acanthuri* Machida and Uchida, 1991 in possessing a smaller ventral sucker (oral sucker width to ventral sucker width ratio 1: 0.5 rather than 1: 1.3-1.7). *Megasolena* sp. n. 1 possesses testes that are contiguous rather than separated as in *Megasolena kyphosi* Sogandares-Bernal, 1959. *Megasolena* sp. n. 1 is further differentiated from *M*. *estrix* in possessing a hermaphroditic sac that is approximately half as large (length and width). No measurement of the hermaphroditic sac of *M*. *estrix* was given by Linton (1910), Manter (1937), or Sogandares-Bernal (1959); thus, comparisons were made based on the illustrations. Eric Pulis and I examined three other specimens of *H*. *ciliaris* collected in the shallow waters (<2 m) off Grassy Key, Florida, but did not recover any haploporoid.

Fam. n. 1

Diagnosis. Body variable in shape, generally elongate. Eyespot pigment usually absent. Tegument thick, with or without spines. Oral sucker subterminal

to terminal. Ventral sucker in anterior third of body, pedunculated or not. Pharynx well-developed. Prepharynx variable, generally less than to equal pharynx length. Oesophagus equal to or longer than pharynx. Caeca cylindrical, terminating blindly near posterior end of body, forming cyclocoel, or uroproct. Testis single, spherical to elongate, smooth to irregular, located in hindbody. Hermaphroditic sac elongate to saccular. External seminal vesicle cylindrical, elongate, generally longer than hermaphroditic sac. Ovary pretesticular in hindbody. Uterus confined to region between ovary and hermaphroditic sac. Eggs operculate or not, filamented or not. Vitellarium with numerous small follicles; with them elongated, coalesced as tubules, or in rosette pattern, usually filling entire available space in hindbody, generally restricted to hindbody. Lymphatic system present or not. Excretory vesicle I-shaped. In marine fishes .

Type-genus: *Hapladena* Linton, 1910 (syns. *Deredena* Linton, 1910; *Hairana* Nagaty, 1948).

Remarks. I consider Fam. n. 1 to include *Hapladena* and *Myodera*, the only members considered in the Megasoleninae with a single testis by Overstreet and Curran (2005a). The name Scorpidicolinae is available; however, *Scorpidicola* was considered a junior synonym of *Myodera* by Overstreet and Curran (2005a), thus a new name is needed. Additionally, members of *Myodera* have an intestine that forms a cyclocoel and is found in kyphosids, thus although I consider it in Fam. n. 1 it may have an association with members of Cadenatellidae. *Hapladena* is chosen as the type genus, as it is more diverse, the only genus to have members with molecular data, and my skepticism that

Myodera forms a natural group with *Hapladena*. Of the haploporoids with a single testis, Fam. n. 1 can be differentiated from the Haploporidae in having a robust tegument (in part), lacking oral lobes (in part), and being in marine fishes, primarily acanthurids, scarids, pomacanthids, and kyphosids. Fam. n. 1 can be differentiated from Cadenatellidae in lacking oral lobes and accessory suckers.

Hapladena acanthuri Siddiqi et Cable, 1960

Type-host: *Acanthurus coeruleus* Bloch et Schneider, blue tang surgeonfish, Acanthuridae.

Other host: *Acanthurus chirurgus* (Bloch), doctorfish, Acanthuridae.

Type-locality: off Parguera, Puerto Rico.

Other locality: off Desecheo Island, Puerto Rico (Dyer et al. 1985); St. Croix, U.S.V.I.

Site: Intestine.

Holotype: USNPC 39346.

Supplemental material: USNM TBD.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-149) from 1 entire dead specimen from St. Croix, U.S.V.I.

Remarks. My specimen matches the description of *A. acanthuri* by Siddiqi and Cable (1960). *Hapladena acanthuri* is differentiated from all *Hapladena* spp. except *Hapladena tanyorchis* Manter et Pritchard, 1961 in possessing a dendritic vitellarium. *Hapladena acanthuri* can be separated from *H*. *tanyorchis* based on

the testis being shorter and a smooth testis rather than irregular and elongate. Additionally, *H. tanyorchis* was described off Hawaii, USA.

Hapladena varia Linton, 1910

Type-host: *Acanthurus chirurgus* (Bloch), doctorfish, Acanthuridae.

Other hosts: *Acanthurus bahianus* Castelnau, ocean surgeonfish; *Acanthurus coeruleus* Bloch et Schneider, blue tang surgeonfish, both Acanthuridae.

Type-locality: off Dry Tortugas, Florida, USA.

Other locality: off Jamaica (Nahhas and Carlson 1994); St. Croix, U.S.V.I.

Site: Intestine.

Syntypes: USNPC 8513, 8514.

Representative DNA sequences: Partial 18S, entire ITS region, partial (D1–D3) 28S: GenBank accession no. TBD (MJA-151) from 1 entire specimen from St. Croix, U.S.V.I.

Remarks: Unfortunately, I do not possess a voucher specimen for *H*. cf. *varia* as only one dead specimen of a large *Hapladena* morphotype was obtained from the intestinal tract of *A. chirurgus*. Based on initial examination, a photomicrograph of the specimen, its final host, and comparison of the specimen in my photomicrograph with the type material, this species is close to *H. varia*. Eric Pulis and I have attempted to collect *H. varia* from its type-host (n=3) near the type-locality (Grassy Key and Marathon, FL) but were unsuccessful.

Hapladena invaginata Caballero, 1987 nom. nud.

Specimen deposited: Institute of Biology, National Autonomous University of Mexico, National Collection of Helminths, Mexico City, Mexico no. 2539.

Remarks. This species was never described (pers. comm. Gerardo Pérez Ponce de León). It was reported by Caballero et al. (1992) and cited by Overstreet et al. (2009). Additionally, based on photomicrographs of the specimen sent by Gerardo Pérez Ponce de León it resembles *H*. *varia*.

Species transferred to other genera

Parasaccocoelium gymnocephali (Sheena et Janardanan, 2007) comb. n.

syn. *Hapladena gymnocephali* Sheena et Janardanan, 2007.

Remarks. Sheena and Janardanan (2007) described and elucidated the life cycle of this species from the Chaliyar and Kadalundi rivers in Kozhikode, India. The first intermediate host is a freshwater gastropod *Gabbia travancorica* (Benson) and the final host is the estuarine perciform *Ambassis gymnocephalus* (Lacepède). The inclusion of fresh and estuarine hosts in the lifecycle, combined with a relatively small body length, restricted uterus, extensive vitelline field, and paired caeca strongly suggest that this species belongs in the Waretrematinae (sensu Overstreet and Curran 2005a, Pulis 2014). *Hapladena gymnocephali* has morphological characters in common with *Parasaccocoelium* Zhukov 1971, a taxon that Overstreet and Curran (2005a) considered a junior synonym of *Pseudohapladena* Yamaguti, 1952 but recently restored by Besprozvannykh et al. (2014). Although Besprozvannykh et al. (2014) consider members of the genus to only infect mugilids, *H. gymnocephali* possesses vitelline fields along the lateral margins that do not unite posttesticularly, a short uterus that contains

few eggs, and a testis that is longitudinally elongate. Host switching within the Waretrematidae seems to be common across genera (Overstreet and Curran 2005a, Pulis et al. 2013, Pulis 2014); thus, I do not consider the host being a perciform rather than a mugilid to be a significant difference for generic differentiation. If this species was left within *Hapladena*, it would represent the only fresh water and estuarine member. Molecular data are needed to confirm the combination of *H. gymnocephali* with *Parasaccocoelium*.

Haploporidae Nicoll, 1914

Diagnosis. Body with variety of shapes. Tegument thin, spinous. Eyespot pigment dispersed in forebody of most adults of all species, distinct in all known cercariae. Oral sucker subterminal or terminal, with or without associated lobes or hood. Ventral sucker in anterior half of body. Prepharynx variable. Pharynx well-developed. Oesophagus variable. Intestine either simple caecum or bifurcated into 2 caeca, with crura sac-like or cylindrical, extending to various levels of body. Testis single, variously shaped. Hermaphroditic sac present, containing internal seminal vesicle, pars prostatica, female duct, and hermaphroditic duct. External seminal vesicle ranging from sac- or club-shaped to uniformly cylindrical and elongate. Genital pore medial, anterior to ventral sucker. Ovary pretesticular, entire. Laurer's canal present. Seminal receptacle present or lacking, with uterine seminal receptacle when lacking. Uterine distribution variable. Eggs with indistinct or distinct operculum; containing miracidia with or without pigmented eyespots. Vitellarium variable in shape and location. Excretory vesicle Y-, I or V-shaped; main stem greatly swollen or not;

pore terminal; concretion(s) present or lacking. Cosmopolitan in intestine of marine, estuarine and freshwater herbivorous fishes, primarily mugilids.

Type-genus: *Haploporus* Looss, 1902.

Remarks. I consider the Haploporidae to contain the subfamilies Haploporinae, Chalcinotrematinae, Forticulcitinae, and Waretrematinae. The Haploporinae can be differentiated from the Atractotrematidae and Megasolenidae in processing a single testis; from the Cadenatellidae in lacking a uroproct; and from the Fam. n. 1 in processing a thin tegument, a generally shorter body, and potentially not infecting acanthurid, scarid, or pomacanthid hosts. *Pholohedra overstreeti* Cribb, Pichelin, et Bray, 1998, tentatively considered a waretrematine, is the only haploporine described from a kyphosid. The host association may indicate that it is better allocated to Fam. n. 1; however, the Waretrematinae also has members that infect scatophagids and have diversified in a variety of fish groups (Overstreet and Curran 2005a, Pulis 2014).

Discussion

Molecular analysis

Based on the results of my phylogenetic analyses, the Haploporidae is paraphyletic if the Atractotrematidae is considered at the family level and the Megasoleninae (sensu Overstreet and Curran 2005a) is considered a subfamily of the Haploporidae. Therefore, I could 1) consider the Atractotrematidae a synonym of the Haploporidae; 2) recognize the Atractotrematidae, Cadenatellidae, and Haploporidae as families but with *Megasolena* and

Hapladena as incertae sedis within the Haploporoidea; or 3) erect separate families for the megasolenines with two testes and with one testis. Durio and Manter (1969) and Ahmad (1985) considered the Atractotrematidae a synonym of the Haploporidae, and Overstreet and Curran (2005b) considered the status of that family as tentative. However, I believe that is an oversimplification of the haploporoids, particularly as the Atractotrematidae + Haploporidae clade was one of the most labile in a phylogeny of the Digenea Carus, 1863 (Olson et al. 2003). Additionally, the inclusion of *Cadenatella* within the Haploporoidea further complicates any decision to consider the Atractotrematidae a junior synonym of the Haploporidae, since the morphological diagnosis would have to include number and arrangement of testes as well as the presence or absence of a hermaphroditic sac. Therefore, I choose to recognize the five main clades at the family level.

The combined hypothesis was very similar to the partial 28S hypothesis, and it was able to resolve the relationship between Megasolenidae and Fam. n. 1. Unfortunately, ITS2 sequences are not available for *H*. *nasonis* and the other two atractotrematids are not available. Therefore, the possibility remains that the addition of those species into the analysis may have a confounding effect on the hypothesis. However, the relatively high support values of the combined BI tree suggest that the ITS2 may be useful in resolving phylogenies that also include 28S rDNA sequence data. Blasco-Costa et al. (2009a) conducted the first two phylogenetic analyses of the Haploporidae: one based on the 28S and one based on the ITS2. Their ITS2 hypothesis did not resolve *Dicrogaster* as

monophyletic, and it had lower support values; however, they did not conduct a combined analysis. The ITS2 is a more variable region than the 28S (e.g., Coleman 2003, Nolan and Cribb 2005) but is less variable and easier to align than the ITS1 (e.g., Nolan and Cribb 2005, Chapter IV). Nolan and Cribb (2005) suggested that the ITS2 may be a useful gene region at the generic level for phylogenies, but Coleman (2003) suggested that it is not a good region for use in phylogenetics at the family, order, or higher levels. Andres et al. (2014b) utilized partial 28S rDNA sequences to generate a molecular hypothesis for the Opecoelidae Ozaki, 1925, and Barnet et al. (2014) used sequences of the ITS2 to do the same, and, although the number of taxa included in both hypotheses was different, the overall topology was very similar. Therefore, the addition of ITS2 sequences may be useful in discriminating the deeper portions of phylogenies (at least at the family level) when used in conjunction with partial 28S rDNA sequences.

Atractotrematidae

Overstreet and Curran (2005b) considered the status of the Atractotrematidae to be tentative, since it contains relatively few dissimilar members and has been previously considered a synonym of the Haploporidae. They recognized *Atractotrema* Goto et Ozaki, 1929, *Isorchis* Durio et Manter, 1969, *Pseudisorchis* Ahmad, 1985, and *Pseudomegasolena* Machida et Kamiya, 1976 in the family but suggested that *Pseudomegasolena* may actually be a megasolenine. I provided molecular data for a species of *Isorchis*, leaving *Pseudisorchis* as the only atractotrematid genus without a member represented

by molecular data. My BI analysis revealed *Isorchis* as the sister to *Pseudomegasolena*, suggesting that having either symmetrical or nearly symmetrical testes is a reliable characteristic for discriminating between haploporoids with two testes. *Pseudisorchis* was erected by Ahmad (1985) for *Isorchis manteri* (Martin, 1973) and is currently a monotypic taxon from the intestine or gall bladder of mugilids off Queensland, Australia. However, Overstreet and Curran (2005a) suggested that what they illustrated as *P. manteri* in their chapter in the *Keys to the Trematoda* Vol. 2 (Figure 13.6) may represent a different species. Once molecular data are available for a member of *Pseudisorchis* that genus will likely be resolved with the other atractotrematids.

Megasolenidae and Fam. n. 1

Manter (1935) was the first to determine a close association between *Megasolena* and *Hapladena*, based on the presence of a hermaphroditic sac, containing lymphatic vessels, and parasitizing marine fishes. Until now, this close association has been maintained by various workers. Blasco-Costa et al. (2009a) suggested that the basal position of the megasolenines relative to the rest of the haploporids may be unreliable as the only representative was *H. nasonis*. They suggested that species may be aberrant because *H. nasonis* is much longer than the rest of the members of *Hapladena*; however, *Hapladena* was resolved as monophyletic with the inclusion of *H. acanthuri* and *H.* cf. *varia*. In my combined tree, *Megasolena* was sister to *Hapladena* and the rest of the haploporoids with a single testis. This position in the tree also seems to have a morphological basis since the atractotrematids also process two testis and are basal to the rest of the

Haploporoidea. Molecular data for members of *Vitellibaculum* will help clarify the interrelationships of the Megasolenidae. No lifecycle information is known for a atractotrematid, nor for a megasolenid. Yamaguti (1975) considered the atractotrematids to belong in a group that contains larvae that encyst in invertebrates. Overstreet and Curran (2005b) questioned this association based on the presumed close relationship to the Haploporidae that encyst on vegetation or occur free in the water as reported by Cable (1962) for a species of *Hapladena*. Based on the phylogeny presented by Olson et al. (2003), the two closest affinities of the Haploporioidea are either the Monorchiata, Cribb, Tkach, Bray et Littlewood, 2003 or Paragonimidae Dollfus, 1939. Members of both of those groups utilize invertebrates as a second intermediate host (Cribb et al. 2003). Thus, although no lifecycle is known for a member in either of the two haploporpoid families with members processing two testes, my determination of the systematic position of the Atractotrematidae and Megasolenidae as basal relative to the haploporoids with a single testis may suggest that the atractotrematids and megasolenids utilize invertebrate intermediate hosts and the single testis haploporoids may not. Additionally, *Vitellibaculum* has members reported from herbivorous fishes as well as members of Lutjanidae and Diodontidae, which are not herbivorous.

Haploporidae

Members of the Haploporidae are more diverse than all of the other known haploporoids combined. Manter (1957) suggested that the diversification of the haploporids was related to the widely distributed mugilid hosts and the ability of

these hosts to live in marine, estuarine, and freshwater. Moreover, some mugilids such as those in the complex of species recognized as *Mugil cephalus* Linnaeus (e.g., Durand et al. 2012a, b, Whitfield et al. 2012) actively seek out freshwater for part of their life history (Whitfield et al. 2012). Manter (1957) considered that the life history of the mugilid hosts could serve as 'ecological bridges' between the three different habitats. Supporting this hypothesis, three of the four haploporid subfamilies have at least one species described from freshwater.

This was the first study to include molecular data for multiple species considered in the Megasoleninae (sensu Overstreet and Curran 2005a), and revealed that the systematic relationships among the marine perciformes haploporoids are more complex than previously thought. However, additional species of haploporoids, and ideally sequences from additional gene regions, need to be examined to determine the validity of the non-Haploporidae families I recognize.

CHAPTER VII

A MULTIGENE HYPOTHESIS OF THE TREMATODE SUPERFAMILY HAPLOPOROIDEA NICOLL, 1914

Abstract

The phylogenetic relationships of species within the Haploporoidea Nicoll, 1914 were assed using Bayesian inference and Maximum Likelihood analysis of partial mitochondrial 12S rRNA, internal transcribed spacer region 2 (ITS2), and partial 28S rRNA sequences as well as a concatenated analysis of those three regions. Sequences of partial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1) were also obtained but not used in the concatenated phylogenetic analysis because they provided the least amount of information. Phylogenetic hypotheses based on partial 12S and ITS2 data alone poorly resolved the relationships of the Haploporoidea when compared with the resolution based on partial 28S and concatenated analyses. The five families of the Haploporoidea recognized in Chapter VI were resolved in the partial 28S and combined trees. The four haploporid subfamilies were also recovered as monophyletic in the28S and concatenated analyses with the exception of the position of *Spiritestis* Nagaty, 1948 in the concatenated analysis. Manter's (1957) hypothesis of species of *Mugil* Linnaeus acting as 'ecological bridges' in the radiation of the Haploporidae is supported based on the topology of my 28S and concatenated tree. Thirteen of the 16 haploporid genera represented in my analysis have at least one member reported from a species of *Mugil*.

Introduction

The Haploporoidea Nicoll, 1914 includes parasites of the alimentary tract and gall bladder of marine, estuarine, and freshwater herbivorous and omnivorous fishes. The superfamily has recently been the subject of several taxonomic revisions, coupled with molecular hypotheses that have revealed the systematics of its members to be more complex than based on morphological characters alone (e.g., Blasco-Costa et al 2009a, Blasco-Costa et al. 2010, Pulis et al. 2013, Pulis 2014, Chapters III, VI). Jones (2005) and Overstreet and Curran (2005a, b) considered the Haploporoidea to include the Atractotrematidae Yamaguti, 1939 and the Haploporidae Nicoll, 1914 (syns. Megasolenidae Manter, 1935, Waretrematidae Srivastava, 1937, Hyporhamphitrematidae Machida et Kuramochi, 2000). Bray et al. (2014) used Bayesian inference (BI) analysis of partial 28S rDNA sequences to demonstrate that *Cadenatella* Dollfus, 1946 was a haploporoid. They considered members of *Cadenatella* to belong in the Cadenatellinae Gibson et Bray, 1982 but based on their BI analysis, did not allocate the subfamily to a family, suggesting that the Cadenatellinae would likely warrant family level status once additional haploporioids were molecularly tested. I used BI of partial 28S rDNA sequences and combined ITS2 and 28S rDNA sequences to show that the Megasoleninae Manter, 1935 and the Haploporidae (sensu Overstreet and Curran 2005a) were paraphyletic (Chapter VI). I elevated the Cadenatellinae to Cadenatellidae Gibson et Bray, 1985, Megasoleninae (minus *Hapladena* Linton, 1910 and *Myodera* Montgomery, 1957) to Megasolenidae, and erected Fam. n. 1 for *Hapladena* and *Myodera* (Chapter VI).

Therefore, the Haploporoidea consists of five families (Chapter VI) and approximately 40 genera (Overstreet and Curran 2005b, Pulis et al. 2013, Pulis 2014, Chapters III-VI).

In all of the previous studies on the haploporids that incorporate molecular data, only nuclear (nc) genes have been used. Mitochondrial (mt) genes have been used extensively in phylogenetic studies of metazoans. For trematodes, mt genes have most often been used to examine intra- and interspecific relationships (e.g., Morgan and Blair 1998, Lockyer et al. 2003,Vilas et al. 2005, Zarowiecki et al. 2007, Králová-Hromadová et al. 2008) and have mostly utilized the cytochrome oxidase 1 (*cox*1) and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad*1). Le et al. (2002) stated that mtDNA sequences are probably of limited value when investigating 'deep-level' phylogenies. Bray et al. (2009) used sequences of *nad*1 gene, in conjunction with partial 28S rDNA sequences to infer the systematic relationships of the Lepocreadioidea Odhner, 1905. The incorporation of sequences of *nad*1 marginally improved their analysis more than the 28S alone; however, they suggested that the *nad*1 gene would not be a mt gene of choice in future studies.

Zarowiecki et al. (2005) examined the mt genome of *Schistosoma* spp. and demonstrated that the lowest nucleotide diversity occurred within sequences of the 12S. Machida et al. (2012) provided a universal primer set for a conserved region of the mt 12S rRNA gene, to expand the number of potential genes available for metazoan metagenetic analyses. They suggested that four such gene regions exist: the nc 18S rRNA, nc 28S, mt *cox*1, and mt 12S rRNA, with

the 18S being the most conserved, followed by the 28S, then the 12S, and finally the *cox*1. The purpose of this study is both to increase the available molecular library for haploporoids and to further resolve the relationships of members of the superfamily.

Materials and Methods

Table 7.1 indicates taxa chosen, the hosts from which they were isolated, and their geographic location. Table 7.2 indicates the GenBank accession numbers for published and new sequences. All ITS2 and 28S rDNA sequences are from previously published studies, with the exception of *Dicrogaster perpusilla* Looss, 1902. Up to fifty putative haploporoidean species were analyzed, representing 5 families and 24 genera. The monorchiids *Hurleytrematoides chaetodoni* (Manter, 1942) and *Lasiotocus haemuli* Overstreet, 1969 were chosen as the outgroup taxa to root the phylogenetic trees.

Table 7.1

Taxa, hosts, and collection locations of trematodes used in this study. Abbreviations GOM: Gulf of Mexico, FL: Florida, MS Mississippi, NC North Carolina, NT Northern Territory, QLD Queensland, WA Western Australia.

Family	Species	Host	Locality
Monorchiidae	*Hurleytrematoides chaetodoni*	*Chaetodon striatus*	St. Croix, U.S.V.I
Monorchiidae	*Lasiotocus haemuli*	*Haemulon sciurus*	Marathon, FL, USA
Atractotrematidae	*Isorchis* cf. *parvus*	*Chanos chanos*	Exmouth Gulf, WA, Australia
Cadenatellidae	*Cadenatella americana*	*Kyphosus sectatrix*	Long Key, FL, USA
Cadenatellidae	*Cadenatella floridae*	*Kyphosus sectatrix*	Long Key, FL, USA
	Cadenatella isuzumi	*Kyphosus vaigiensis*	Heron Island, QLD, Australia
	Cadenatella pacifica	*Kyphosus vaigiensis*	Heron Island, QLD, Australia
Fam. N. 1	*Hapladena acanthuri*	*Acanthurus chirurgus*	St. Croix, U.S.V.I
Fam. N. 1	*Hapladena* cf. *varia*	*Acanthurus chirurgus*	St. Croix, U.S.V.I
Megasolenidae	*Megasolena hysperospina*	*Archosargus rhomboidalis*	Marathon, FL, USA

Table 7.1 (continued).

Family	Species	Host	Locality
Megasolenidae	*Megasolena* sp. n.	*Holacanthus ciliaris*	West Florida Middle Grounds, GOM
Haploporidae			
Chalcinotrematinae	*Chalcinotrema elongata*	*Prochilodus lineatus*	Rio de La Plata, Argentina
Chalcinotrematinae	*Chalcinotrema magna*	*Cyphocarynx voga*	Rio de La Plata, Argentina
Chalcinotrematinae	*Intromugil alachuaensis*	*Mugil cephalus*	Santa Fe River, FL, USA
Chalcinotrematinae	*Intromugil mugilicolus*	*Mugil cephalus*	Ocean Springs, MS, USA
Chalcinotrematinae	*Saccocoelioides beauforti* sensu stricto	*Mugil cephalus*	Wilmington, NC, USA
Chalcinotrematinae	*Saccocoelioides beauforti* sensu lato	*Mugil cephalus*	Ocean Springs, MS, USA
Chalcinotrematinae	*Saccocoelioides cichlidorum*	*Hypsophyrys nicaraguensis*	Rio Animas, Costa Rica
Chalcinotrematinae	*Saccocoelioides nanii*	*Prochilodus lineatus*	Los Talas, Argentina
Forticulcitinae	Gen. n. 1 *fastigata*	*Mugil cephalus*	Ocean Springs, MS, USA
Forticulcitinae	*Forticulcita* sp. n. 1	*Mugil liza*	Rio de La Plata, Argentina
Forticulcitinae	*Forticulcita* sp. n. 2	*Mugil cephalus*	Salt Springs, FL, USA
Haploporidae	*Dicrogaster contracta*	*Liza aurata*	Santa Pola, Spain
Haploporidae	*Dicrogaster perpusilla*	*Mugil cephalus*	Crete, Greece
Haploporidae	Gen. n. 2 magnisaccus	cf. *Chelon subviridis*	Nha Trang, Vietnam
Haploporidae	Gen. n. 2 megasacculum	cf. *Chelon subviridis*	Nha Trang, Vietnam
Haploporidae	Gen. n. 2 mugilis	cf. *Chelon subviridis*	Daya Bay, China
Haploporidae	Gen. n. 2 sp. n. 1	*Valamugil buchanani*	Broome, WA, Australia
Haploporidae	Gen. n. 2 sp. n. 2	*Paramugil georgii*	Barred Creek, WA, Australia
Haploporidae	Gen. n. 2 sp. n. 3	*Moolgarda perusii*	Fannie Bay, NT, Australia
Haploporidae	Gen. n. 2 sp. n. 4	*Moolgarda seheli*	Hervey Bay, QLD, Australia
Haploporidae	*Litosaccus brisbanensis*	*Mugil cephalus*	Shorncliffe, QLD, Australia
Haploporidae	*Pseudodicrogaster* sp. n. 1	*Ellochelon vaigiensis*	Fannie Bay, NT, Australia
Haploporidae	*Pseudodicrogaster* sp. n. 2	*Ellochelon vaigiensis*	Broome, WA, Australia
Haploporidae	*Pseudodicrogaster* sp. n. 3	*Ellochelon vaigiensis*	Withnell Bay, WA, Australia
Waretrematinae	*Capitimitta costata*	*Selenotoca multifasciata*	Shorncliffe, QLD, Australia
Waretrematinae	*Capitimitta darwinensis*	*Selenotoca multifasciata*	Fannie Bay, NT, Australia
Waretrematinae	*Carassotrema estuarinum*	*Mugil cephalus*	Daya Bay, China
Waretrematinae	*Carassotrema* sp. 2	*Carassius auratus*	Shaoguan, China

Table 7.1 (continued).

Family	Species	Host	Locality
Waretrematinae	Gen. sp.	*Ellochelon vaigiensis*	Broome, WA, Australia
Waretrematinae	*Malabarotrema lobolecithum*	*Chelon subviridis*	Exmouth Gulf, WA, Australia
Waretrematinae	*Malabarotrema megaorchis*	*Mugil cephalus*	Daya Bay, China
Waretrematinae	*Malabarotrema* sp. 1	*Chelon subviridis*	Exmouth Gulf, WA, Australia
Waretrematinae	*Spiritestis herveyensis*	*Moolgarda seheli*	Hervey Bay, QLD, Australia
Waretrematinae	*Spiritestis herveyensis*	*Chelon subviridis*	Exmouth Gulf, WA, Australia
Waretrematinae	*Unisaccoides vitellosus*	*Chelon subviridis*	Beelbi Creek, GLD, Australia
Waretrematinae	*Unisaccus brisbanensis*	*Chelon subviridis*	Hervey Bay, QLD, Australia
Waretrematinae	*Unisaccus* sp. 2	*Chelon subviridis*	Withnell Bay, WA, Australia

Table 7.2

GenBank accession numbers of taxa used in this study.

	Gene region and GenBank accession numbers			
Species	ITS2	28S	12S	NADH
Hurleytrematoides chaetodoni	MJA-608	MJA-608	MJA-608	MJA-608
Lasiotocus haemuli	MJA-110	MJA-110	MJA-110	MJA-110
Isorchis cf. *parvus*	EP-86	EP-86	EP-86	EP-86
Cadenatella americana	MJA-891	MJA-891	MJA-891	-
Cadenatella floridae	MJA-815	MJA-815	MJA-815	MJA-815
Cadenatella isuzumi	-	-	-	FJ788456
Cadenatella pacifica	-	-	-	FJ788456
Hapladena acanthuri	MJA-149	MJA-149	MJA-149	MJA-149
Hapladena cf. *varia*	MJA-151	MJA-151.	MJA-151	MJA-151
Megasolena hysperospina	EP-625	EP-625	EP-625	EP-625
Megasolena sp. n. 1	MJA-735	MJA-735	MJA-735	MJA-735
Chalcinotrema elongata	SSC-37	SSC-37	SSC-37	SSC-37
Chalcinotrema magna	SSC-39	SSC-39	SSC-39	SSC-39
Intromugil mugilicolus	KC430096	KC430096	EP-6	EP-6
Saccocoelioides beauforti sensu stricto	MJA-260	MJA-260	MJA-260	MJA-260
Saccocoelioides beauforti sensu lato	MJA-719	MJA-719	MJA-719	MJA-719
Saccocoelioides cichlidorum	EP-396	EP-396	EP-396	-
Saccocoelioides nanii	EP-344	EP-344	EP-344	EP-344
Gen. n. 1 *fastigata*	MJA-281	MJA-281	MJA-281	MJA-281
Forticulcita sp. n. 1	SSC-23	SSC-23	SSC-23	EP_339
Forticulcita sp. n. 2	SJC3-1	SJC3-1	SJC3-1	SJC3-1
Dicrogaster perpusilla	DNA-629	DNA-629	DNA-626	DNA-629
Gen. n. 2 *magnisaccus*	MJA-769	MJA-769	MJA-769	MJA-769
Gen. n. 2 *megasacculum*	MJA-767	MJA-767	MJA-767	-

Table 7.2 (continued).

Species	Gene region and GenBank accession numbers ITS2	28S	12S	NADH
Gen. n. 2 *mugilis*	EP-610	EP-610	EP-005	-
Gen. n. 2 sp. n. 1	MJA-303	MJA-303	MJA-303	MJA-303
Gen. n. 2 sp. n. 2	EP-561	EP-561	EP-561	EP-561
Gen. n. 2 sp. n. 3	EP-165	EP-165	EP-165	EP-165
Gen. n. 2 sp. n. 4	EP-154	EP-154	EP-154	EP-154
Litosaccus brisbanensis	KM253765	KM253765	EP-161	EP-161
Pseudodicrogaster sp. n. 1	EP-251	EP-251	MJA-355	MJA-355
Pseudodicrogaster sp. n. 2	EP-126	EP-126	EP-126	MJA-661
Pseudodicrogaster sp. n. 3	MJA-885	MJA-885	MJA-885	MJA-885
Capitimitta costata	KC206497	KC206497	EP-219	EP-219
Capitimitta darwinensis	KC206498	KC206498	MJA-370	MJA-370
Carassotrema estuarinum	EP-198	EP-198	EP-198	EP-198
Carassotrema sp. 2	EP-190	EP-190	EP-190	-
Gen. sp.	MJA-366	MJA-366	MJA-366	MJA-366
Malabarotrema lobolecithum	EP-568	EP-568	MJA-232	MJA-232
Malabarotrema megaorchis	EP-644	EP-644	EP-644	-
Malabarotrema sp. 1	EP-148	EP-148	MJA-231	MJA-231
Spiritestis herveyensis	KC206500	KC206500	MJA-234	MJA-234
Unisaccoides vitellosus	EP-379	EP-379	EP-379	EP-379
Unisaccus brisbanensis	EP-376	EP-376	EP-376	EP-376
Unisaccus sp. 2	EP-591	EP-591	EP-591	EP-591

Table 7.3

Primers used for amplification and sequencing of mitochondrial 12S and nad1 genes, references for primers and PCR protocols, and approximate PCR product length; all primers are 5'-3'. Abbreviations: F, Forward, R, Reverse, ITS, Internal Transcribed Spacer region, bp, base pairs.

Region Primer name	Direction	Primer sequence	Approximate PCR product length	Reference
partial 12S			ca. 370 bp	
12SF	F	GTGCCAGCHNHHGCGGTYA		Machida et al. 2012
12SR	R	RRRDYGACGGGCR RTDTGT		
partial *nad*1			ca. 470 pb	Bray et al. 1999
JB11	F	AGATTCGTAAGGGGCCTAATA		Morgan and Blair 1998
ND1J2a	R	CTTCAGCCTCAGCATAATC		Bray et al. 1999
partial 18S, ITS1, 5.8, ITS2, partial 28S			ca 2,500 bp	Tkach et al. 2003
ITSF	F	CGCCCGTCGCTACTACCGATTG		
1500R	R	GCTATCCTGAGGGAAACTTCG		

Table 7.3 (continued).

Region Primer name	Direction	Primer sequence	Approximate PCR product length	Reference
Additional primers for sequencing reaction				
DIGL2	F	AAGCATATCACTAAGCGG		
300F	F	CAAGTACCGTGAGGGAAAGTTG		
900F	F	CCGTCTTGAAACACGGACCAAG		
300R	R	CAACTTTCCCTCACGGTACTTG		
DIGL2R	R	CCGCTTAGTGATATGCTT		
ECD2	R	CTTGGTCCGTGTTTCAAGACGGG		

Genomic DNA was isolated using Qiagen DNAeasy Tissue Kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer's instructions. Primers used for PCR and sequencing reactions, references for primers and thermocycler settings, and approximate size of PCR products are in Table 7.3. The resulting PCR products were excised from PCR gel using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, California, USA) following the kit instructions, cycle-sequenced using ABI BigDye™ chemistry (Applied Biosystems, Inc., Carlsbad, California, USA), ethanol-precipitated, and run on an ABI 3130 Genetic Analyzer™. Contiguous sequences were assembled using Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, USA, Version 5.0). Sequences of the four gene regions were aligned using MAFFT version 6.611b (Katoh et al. 2005) with 1000 cycles of iterative refinement and the *genafpair* algorithm. The alignment of ITS2, partial 28S (henceforth just 28S), and partial 12S (henceforth just 12S) gene sequences were masked with ZORRO (Wu et al. 2012) using default settings, positions with confidence scores <0.4 were excluded. All alignments were trimmed to the shortest sequence on both 5' and 3' ends in BioEdit, ver. 7.1.3.0. (Hall 1999).

Phylogenetic analysis of the data was performed using BI and Maximum Likelihood (ML). The best nucleotide substitution model for both methods and for each gene region was estimated with jModeltest-2 (Darriba et al. 2012). The best model for the 28S and *nad*1 analyses was the general time reversible with estimates of invariant sites and gamma-distributed among site-rate variation (GTR + I + Γ). The best model for the ITS2 was the GTR + Γ. The best model for the 12S analysis was the transversion model (TVM) + I + Γ, but because of the program limitations, the GTR + I + Γ model was used.

Bayesian inference was performed with MrBayes 3.1.2 software (Huelsenbeck and Ronquist 2001). The following model parameters were used in MrBayes: ngen = 1,000,000 and samplefreq = 100 for single gene hypotheses. Burn-in value was 1,500 estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values (sump burnin = 1,500), and nodal support was estimated by posterior probabilities (sumt) (Huelsenbeck et al. 2001) with all other settings left as default. For the concatenated analysis: ngen = 4,000,000, samplefreq =1,000, burnin values = 1,000.

Maximum Likelihood was performed using RAxML (Stamatakis et al. 2005), with the rapid Bootstrap analysis and search for best-scoring ML tree in one run (-f a). Nodal support was estimated by ML bootstrapping, utilizing the –B option in RAxML for ‘Bootstopping’ (Pattengale et al. 2010) with the default 0.03 bootstopping criterion. Clades were considered to have high nodal support if BI posterior probability (pp) was ≥95% and ML bootstrap resampling (bsp) was ≥70%. For the concatenated BI and ML analyses of the ITS2, 28S, and 12S, the

partitions used correspond with the three gene regions and implement the same nucleotide substitution model.

Substitution saturation of *nad*1 sequences was evaluated by estimating the index of substitution saturation (Iss) (Xia et al. 2003) in DAMBE 5.3 (Xia 2013). A partition homogeneity test (incongruence length difference test; Farris et al. 1995) was conducted using PAUP* (Swofford 2002) to determine whether the ITS2, 28S, and 12S gene regions were significantly heterogeneous from each other. Shimodaira-Hasegawa (S-H) tests were run in RAxML to compare the concatenated gene tree with the individual gene trees, using likelihood parameters taken from the individual ML analyses. Trees are displayed on the BI analysis, with posterior probabilities displayed above ML bootstrap resampling. In cases where BI analysis recovered a polytomy, the polytomy is left, but no support values are provided.

Results

Sequences of *nad*1 were found to be saturated at the third codon position ($p = 0.001$; the $I_{ss} > I_{ss.c}$ if the true tree was both symmetrical and asymmetrical), thus the BI analysis (Figure 7.1) excluded the third codon position. Additionally, nad1 sequences could not be obtained from all species treated, thus *nad*1 sequences were excluded from the concatenated analyses. The BI analysis resolved one of the outgroup species with the ingroup and had many erroneous groupings (e.g., *Cadenatella floridae* Overstreet, 1969 outside of all other members except *Malabarotrema lobolecithum* (Martin, 1973) and the other *Cadenatella* spp. + the monorchiid *Lasioticus haemuli* as sister to a clade of

Carassotrema sp. 2 and Gen. n. 2 sp. 4 + *Spiritestis herveyensis* Pulis et Overstreet, 2012).

The partition homogeneity test indicated that 12S was heterogeneous (p = 0.01) from both the ITS2 and 28S data; and that the ITS2 was heterogeneous (p = 0.01) from the 28S. Although all three regions were heterogeneous, I still present a concatenated analysis but present the three individual hypotheses as well.

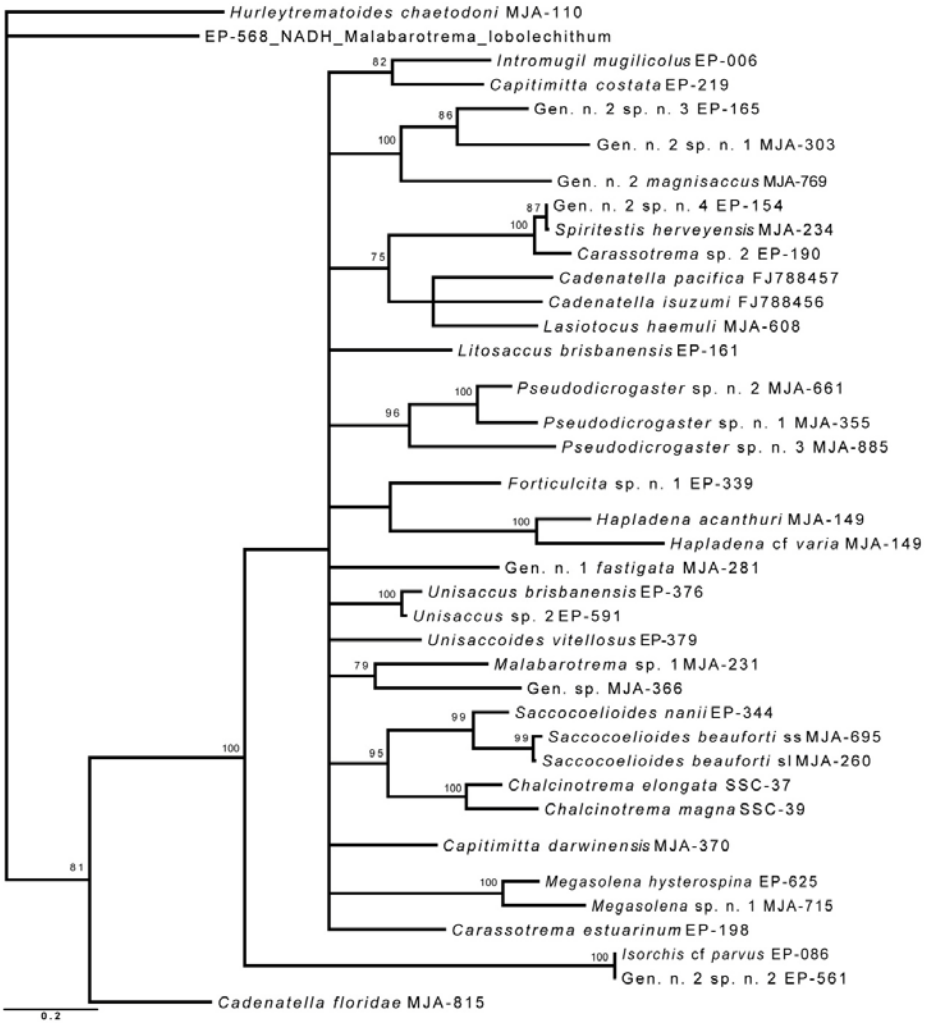


Figure 7.1. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial *nad*1 sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100).

28S Figure 7.2.

The 28S BI and ML topologies were almost identical and generally characterized by high nodal support. The ML analysis resolved Fam. n. 1 as sister to Cadenatellidae with high (73% bsp) nodal support, and Cadenatellidae as sister to Haploporidae with low (43% bsp) nodal support. The four haploporine subfamilies were recovered as monophyletic; however, *Intromugil* Overstreet et Curran, 2005 was not strongly supported as sister to the rest of the Chalcinotrematidae.

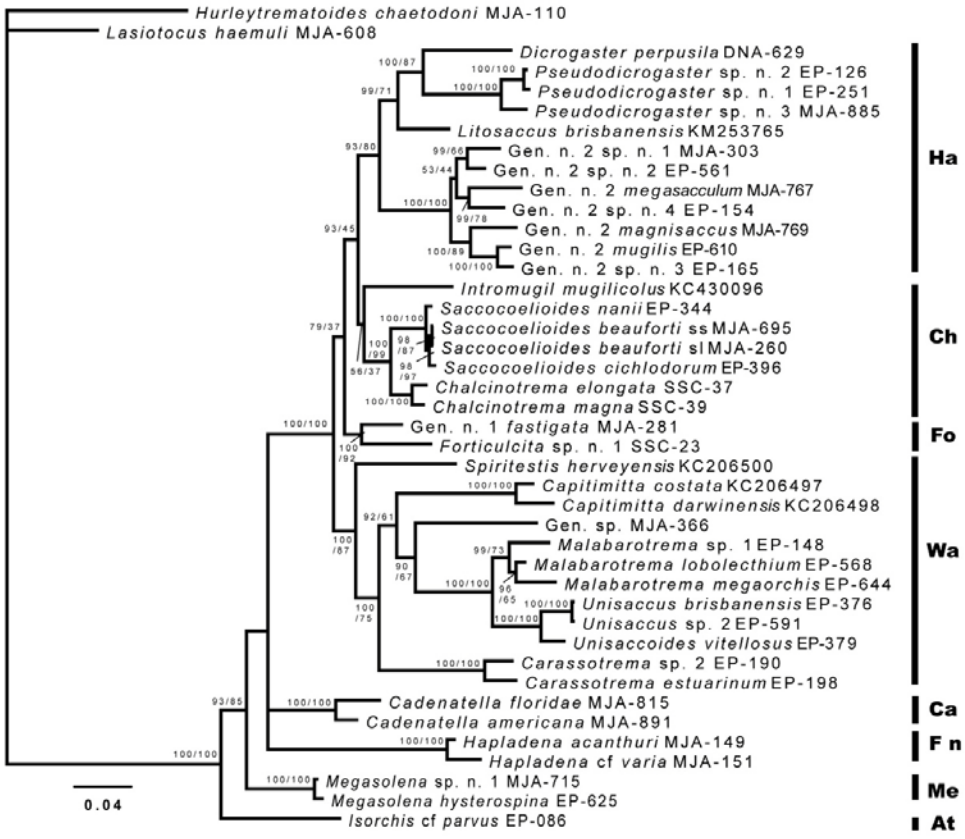


Figure 7.2. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 28S rDNA sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100). Support values as posterior probability percent/ bootstrap resampling percent. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellidae; Ch = Chalcinotrematinae; F n = Fam. n. 1; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasolenidae; Wa = Waretrematinae.

ITS2 Figure 7.3.

The ITS2 BI and ML topologies were also similar, but both were characterized by mostly poor support. The only family resolved as monophyletic was Cadenatellidae. The Haploporinae was paraphyletic, and surprisingly Fam. n. 1 and *Isorchis* Durio et Manter, 1969 were resolved in an unresolved clade with *Pseudodicrogaster* spp. *Intromugil* was not resolved with the Chalcinotrematinae, and *S. herveyensis* was not resolved with the Waretrematinae. Otherwise, the Waretremtinae and Chalcinotrematinae were strongly supported. Members of the haploporine Gen. n. 2 were better resolved than in previous studies (Chapter V, VI), while members of the closely related waretrematine genera *Malabarotrema* Zhukov, 1972, *Unisaccus* Martin, 1973, and *Unisaccoides* Martin, 1973 were not.

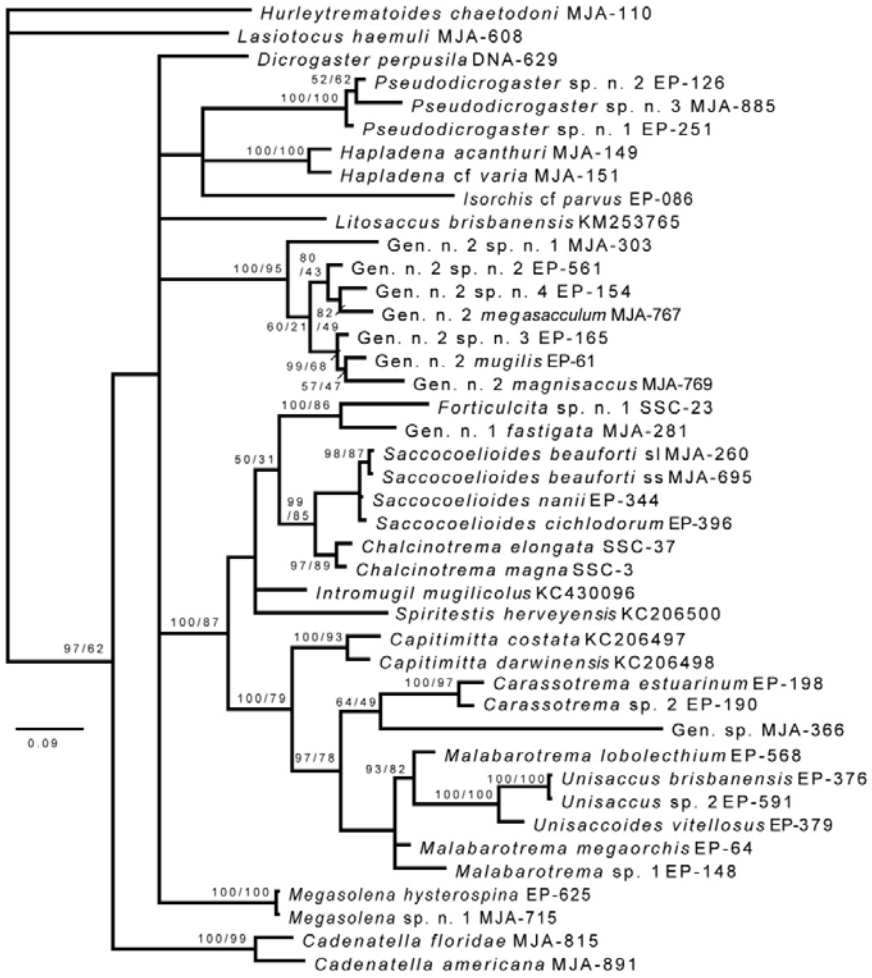


Figure 7.3. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of Internal Transcribed Spacer Region 2 rDNA gene sequences (GTR + Γ, 1,000,000 generations and a sample frequency of 100). Support values as posterior probability percent/ bootstrap resampling percent. Vertical bars denote family or subfamily groups.

12S Figure 7.4.

The 12S BI and ML topologies were also similar, but both were characterized by mostly poor support. The Atractotrematidae, Megasolenidae, and Fam. n. 1 were recovered as a polytomy sister to the Cadenatellidae. The ML topology revealed Fam. n. 1 as the poorly supported (22% bsp) sister to the Atractortrematidae + Megasolenida, also with poor (26% bsp) nodal support. *Spiritestis herveyensis* was resolved as the sister to the rest of the Haploporidae

with good support (93 pp) in the BI analysis but poor support (40% bsp) in ML. *Intromugil* was resolved as the poorly supported sister to the rest of the Chalcinotrematinae in the BI analysis, but as the poorly supported (11% bsp) sister to Forticulcitinae + Chalcinotrematinae in the ML analysis. In both estimation methods *Capitimitta* Pulis et Overstreet, 2012 was resolved outside of the rest of the non-*Spiritestis* waretrematines. The Haploporinae was recovered as monophyletic but with poor support (an unresolved polytomy in BI analysis and nodal supports of less than 33% bsp in ML analysis).

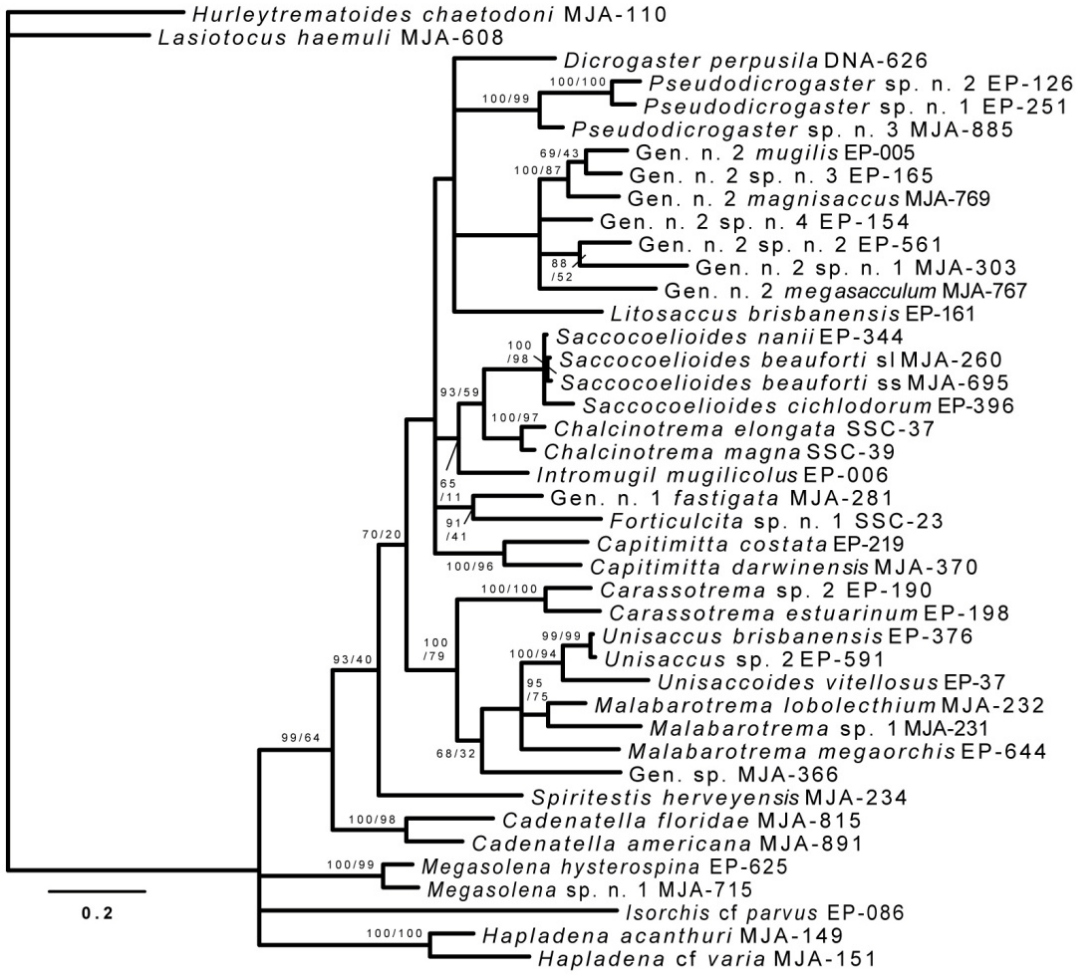


Figure 7.4. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis of partial 12S rDNA sequences (GTR + I + Γ, 1,000,000 generations and a sample frequency of 100). Support values as posterior probability percent/ bootstrap resampling percent.

Concatenated Figures 7.5-6.

The S-H tests revealed the ITS2 and 12S topologies to be significantly different ($p < 0.01$) than the concatenated tree, while the 28S was not. The concatenated BI and ML topologies were nearly identical and characterized by mostly strong support. The five families were resolved as monophyletic and with strong support for all, except the position of Fam. n. 1 as sister to the Cadenatellidae + Haploporidae. The four haploporid subfamilies were resolved as monophyletic, with the exception of *S*. *herveyensis* with the Waretrematinae. The BI analysis recovered *S*. *herveyensis* in a poltyomy with the Waretrematinae and Forticulcitinae + Chalcinotrematinae. In the ML analysis *Spiritestis* was the poorly supported sister to the Forticulcitinae. Thirteen of the 16 haploporid genera treated in my analyses have at least one member reported from a species of *Mugil* (Figure 7.6).

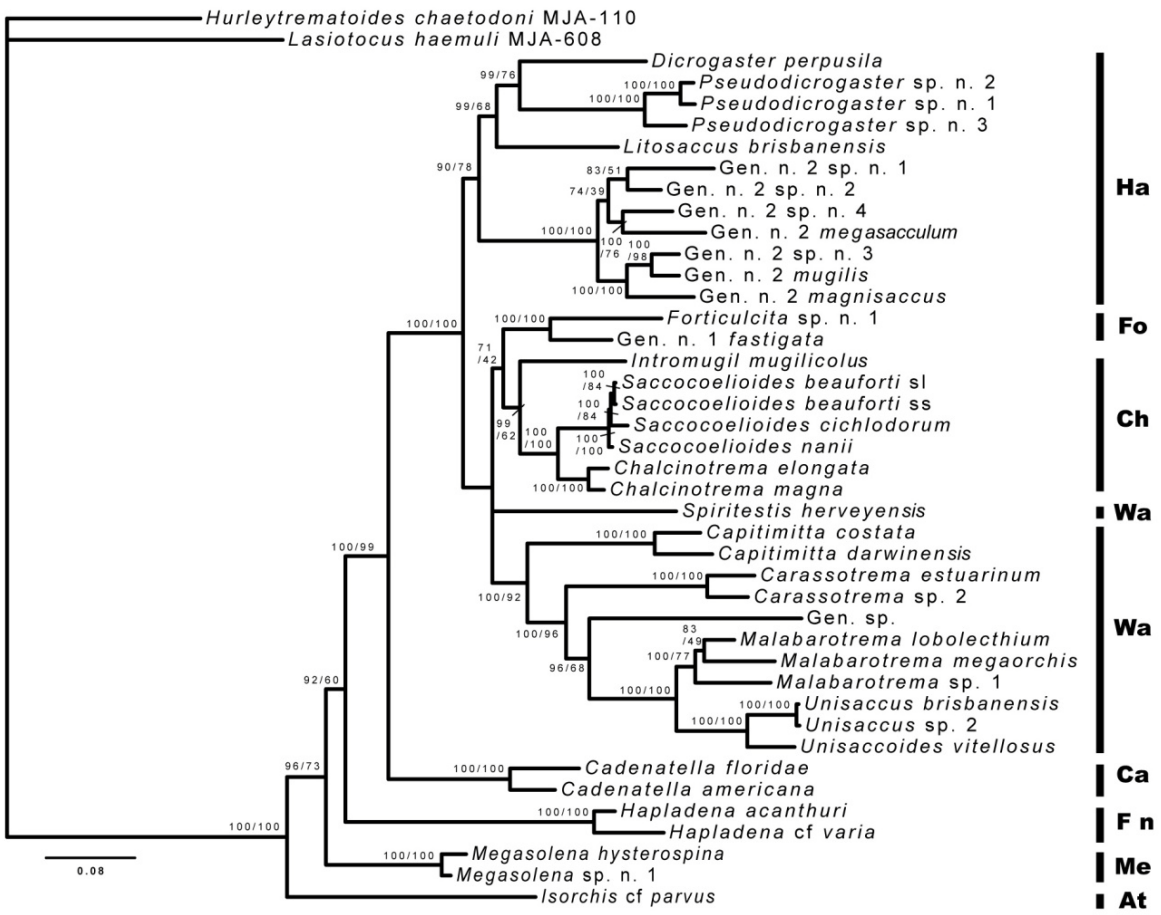


Figure 7.5. Phylogenetic relationships among members of the Haploporoidea resulting from Bayesian inference analysis and Maximum Likelihood of concatenated partial 28S, Internal Transcribed Spacer Region 2 (ITS2), and 12S sequences (28S and 12S GTR + I + Γ; ITS2 GTR + Γ, 4,000,000 generations and a sample frequency of 1,000). Support values as posterior probability percent/ bootstrap resampling percent. Vertical bars denote family or subfamily groups. At = Atractotrematidae; Ca = Cadenatellidae; Ch = Chalcinotrematinae; F n = Fam. n. 1; Fo = Forticulcitinae; Ha = Haploporinae; Me = Megasolenidae; Wa = Waretrematinae.

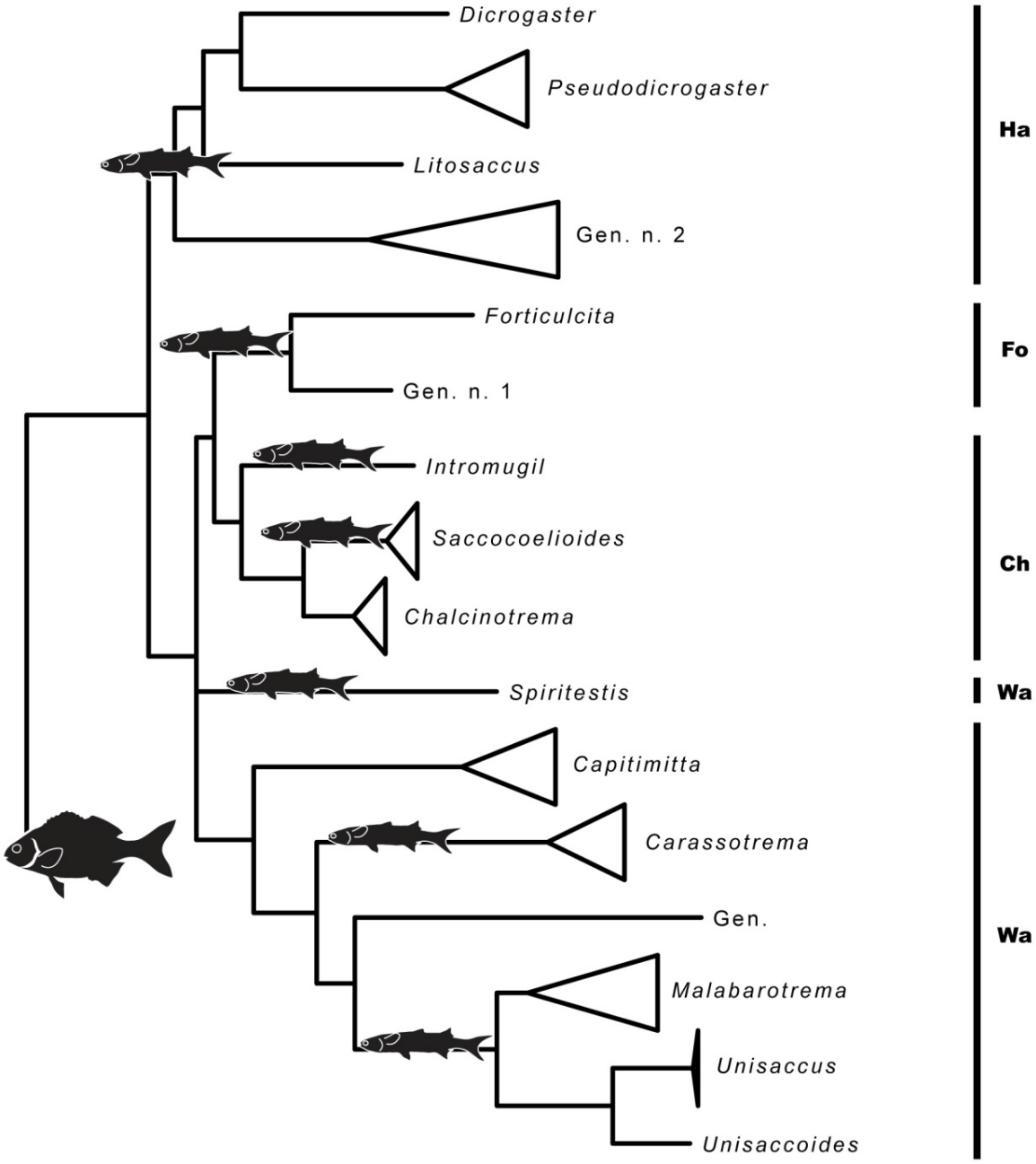


Figure 7.6. Concatenated 28S, ITS2, and 12S gene tree, showing just the Haploporidae with species collapsed to generic level. Mullet superimposed over branches or nodes indicate genera that have at least one species that parasitize species of *Mugil*. The kyphosid represents the branch leading to the marine fish haploporoids. Vertical bars denote subfamily groups. Ch = Chalcinotrematinae; Fo = Forticulcitinae; Ha = Haploporinae; Wa = Waretrematinae.

Discussion

Although the three gene regions used in the concatenated analysis were found to be heterogeneous, the concatenated tree yielded the best supported topology. The five families proposed in Chapter VI were supported, and for the most part strongly supported. The only discrepancy between the concatenated hypothesis and the 28S hypothesis was the placement of *Spiritestis* Nagaty, 1948. In the concatenated ML analysis *Spritestis* was poorly supported as the sister to the Forticulcitinae, while the BI analysis included *Spiritestis* in a polytomy with Forticulcitinae + Chalcinotrematinae and Waretrematinae. In the 28S only analysis, *Spiritestis* was sister to the rest of the waretrematines. In previous phylogenetic analyses using BI of partial 28S rDNA sequences, *Spiritestis* was one of the most labile taxa (see Pulis and Overstreet 2013, Besprozvannykh et al. 2014, Bray et al. 2014, Andres et al. 2014a, Chapters III-VI). Pulis and Overstreet (2013) suggested that *Spiritestis* may occupy a basal position within the Waretrematinae, as members of the genus possess morphological features in common (e.g., elongated testis, ornamented oral sucker, elongated external seminal vesicle) with the Megasoleninae *sensu* Overstreet and Curran (2005a). The labile position of *Spiritestis* may suggest that it will eventually require a separate subfamily to accommodate it, but I refrain from doing so at this time. Particularly, as *Waretrema* Srivastava, 1937 does not have a representative with molecular data.

In the combined analysis (Figure 7.5), the Forticulcitinae and Chalcinotrematinae are sister to each other, although with poor support,

compared with the 28S topology where the Forticulcitinae, then the Chalcinotrematinae, and then the Haploporinae serially branch off from each other. The Forticulcitinae is the most underrepresented haploporid subfamily included in my analyses. In Chapter III, I suggest that the subfamily has a New World origin, so the potentially close relationship between its members and the almost entirely New World Chalcinotrematinae (Overstreet and Curran 2005a), as resolved in the combined tree, might be plausible. *Intromugil* has been labile in most analyses (see Bray et al. 2014, Pulis 2014, Andres et al. 2014a), although always close with the only chalcinotrematine used in those analyses. Pulis (2014) formally suggested that *Intromugil* be moved to the Chalcinotrematinae. However, additional New World, non-*Saccocoelioides* species incorporated into a molecular framework may show that *Intromugil* represents a distinct haploporid lineage. In the concatenated analysis *Intromugil* was strongly supported by BI as the sister to the other chalcinotrematines, but *Intromugil* was poorly supported in the 28S only tree.

Manter (1957) suggested that the diversity of haploporids in estuarine and freshwater environments was related to their mugilid hosts being widely distributed and the affinity that some mullet have for freshwater. He considered the hosts' ability to survive in freshwater, estuarine, and marine habitats as a means of dispersal, or 'ecological bridge,' that the haploporids could use to exploit other fishes with similar feeding habits. In particular, the flathead grey mullet, *Mugil cephalus* Linnaeus species complex (e.g., Durrand et al. 2012a, b), which may consist of up to 14 closely related species, has a high affinity for

freshwater (Whitfield et al. 2012). Members of *Mugil*, in particular *M*. *cephalus*, are widely reported as hosts for species in many haploporid genera. When that host association is mapped on to the concatenated phylogeny (Figure 7.6), it supports Manter's (1947) hypothesis that the radiation of the haploporids is related to that of *Mugil*. In particular, 13 of the 16 genera treated have at least one species described or reported from a species of *Mugil*. Every haploporine genus, with the exception of the monotypic *Ragaia* Blasco-Costa, Montero, Gibson, Balbuena, et Kostadinova, 2009, has at least one species reported from *Mugil* (including the three genera from the Mediterranean Sea not included in the concatenated analysis; see below). The Waretrematinae and Chalcinotrematinae both have members that parasitize non-mugilid fishes. If the position of *Intromugil* in concatenated tree represents the 'true' position, and *Spiritestis* is a basal waretrematine (as Pulis and Overstreet 2013 suggest), then the sister group both of those radiations is hosted by species of *Mugil*. Unfortunately, those two haploporid genera have been the most labile. Additionally, mullet have historically had confusing taxonomic histories and can be a challenge to identify because of the similarity of morphological characters used to define taxa within the Mugilidae Cuvier (e.g., Thomson 1997, Durrand et al. 2012, Whitfield et al. 2012). Therefore, some of the haploporid species that are reported from *M*. *cephalus* sensu lato may be a consequence of host misidentification.

I included in my analyses only those haploporoid species that possessed sequences for all three gene regions. Blasco-Cosata et al. (2009a) provided sequences of the ITS2 and partial 28S rRNA regions, but I refrained from

including them in analyses because each species was also missing between 6-151 bp in the 3' end of their 28S sequences (correlating approximately to the D1 of 28S rRNA). Missing data may (e.g., Grievink et al. 2013) or may not (e.g., Wiens and Morrill 2011) be problematic when resolving phylogenies, but I considered the missing portions of the 28S to be significant since the most informative region was the 28S.

I attempted unsuccessfully to amplify the *cox*1 using the primer sets reported by Morgan and Blair (1998), Králová-Hromadová et al. (2008), and Moszczynska et al. (2009). Only the primers used by Králová-Hromadová et al. (2008) would yield a PCR product in some of the haploporoids tested but sequences of those PCR products were short and would only extend approximately 40 bp on the 3" and 5" end of the *cox*1. Attempts to design PCR primers based on those products have been unsuccessful. Finding a universal primer for the *cox*1 has proven difficult to find for some groups of metazoans (Machida et al. 2012); but the region has shown promise in elucidating inter- and intraspecific relationships (e.g., Vilas et al. 2005). An effort to generate a complete *cox*1 for the haploporoids is ongoing in hopes of further expanding the 'molecular toolbox' for the Haploporoidea.

This is the first phylogenetic study that I am aware of to attempt to utilize the 12S to examine more than two closely related trematode species. I found that the phylogenetic signal of the 12S was not as strong as the 28S, a finding that was expected since the 12S is a mt gene (e.g., Le et al. 2002, Nolan and Cribb 2005). However, the 12S did have more of a phylogenetic signal than both the

*nad*1 and ITS2. Therefore, I would encourage future workers to further explore this gene at the generic to subfamily level. Waeschenbach et al. (2012) utilized a contiguous fragment of mt genome data spanning ca. 4,000 -4,400 bp to investigate the ordinal level relationships of cestodes. They found a conflicting signal between mt and nc gene analyses; however, a similar approach with the haploporids may be useful. Mitochondrial gene order is another potential method for resolving the relationships of the Haploporoidea, as it has been shown to provide a similar topology for the schistosome blood flukes as a nc rRNA hypothesis (Webster and Littlewood 2012).

This study represents the first use of mt markers for investigating the relationships of members of the Haploporoidea. The *nad*1 had an extremely limited phylogenetic signal, but it might be useful for resolving closely related species when excluded from a broader phylogenetic picture. Similarly, the 12S and ITS2 were capable of resolving some but not all interspecific relationships. Thus, the intra- and inter-specific variability of the *nad*1 and 12S needs to be investigated for the haploporoids.

APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICES OF COMMITTEE ACTION

The University of
Southern Mississippi

Institutional Animal Care and Use Committee

118 College Drive #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **07092701**
PROJECT TITLE: **Collection, Maintenance, Life Histories, Taxonomy, & Experimental Studies With Parasites, Disease-Causing Agents, & Host Biology of Fishes**
PROPOSED PROJECT DATES: **2005 - 2010**
PROJECT TYPE: **Project Renewal**
PRINCIPAL INVESTIGATOR(S): **Robin M. Overstreet, Ph.D.**
COLLEGE/DIVISION: **College of Marine Science**
DEPARTMENT: **Coastal Sciences**
FUNDING AGENCY/SPONSOR: **National Science Foundation, PEET & SGER, U.S. Department of Commerce, BCARC, U.S. Department of Agriculture CSREES, U.S. Marine Shrimp Farming Program**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
EXPIRATION DATE: **September 30, 2010**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

9-27-07
Date

The University of
Southern Mississippi

Institutional Animal Care and Use Committee

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www.usm.edu/spa/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: **10100105**
PROJECT TITLE: **Collection, Maintenance, Life Histories, Taxonomy, and Experimental Studies with Parasites, Disease-Causing Agents, and Host Biology of Fishes**
PROPOSED PROJECT DATES: **10/01/2010 to 09/30/2013**
PROJECT TYPE: **New Project**
PRINCIPAL INVESTIGATOR(S): **Robin M. Overstreet, Ph.D.**
COLLEGE/DIVISION: **College of Science & Technology**
DEPARTMENT: **Coastal Sciences**
FUNDING AGENCY/SPONSOR: **National Science Foundation, National Marine Fisheries Service, National Oceanic & Atmospheric Administration, Departmental Awards**
IACUC COMMITTEE ACTION: **Full Committee Review Approval**
PROTOCOL EXPIRATION DATE: **09/30/2013**

Robert C. Bateman, Jr., Ph.D.
IACUC Chair

9-14-2010
Date

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.4063 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**10100105**
PROJECT TITLE:	**Collection, Maintenance, Life Histories, Taxonomy, and Experimental Studies with Parasites, Disease-causing Agents, and Host Biology of Fishes**
PROPOSED PROJECT DATES:	**9/2013 – 9/ 2015**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Robin Overstreet**
DEPARTMENT:	**Coastal Sciences**
FUNDING AGENCY/SPONSOR:	**US Fish & Wildlife Service, USM Development Accts.**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2015**

F Moore 11-11-13

Frank Moore, Ph.D. Date
IACUC Chair

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